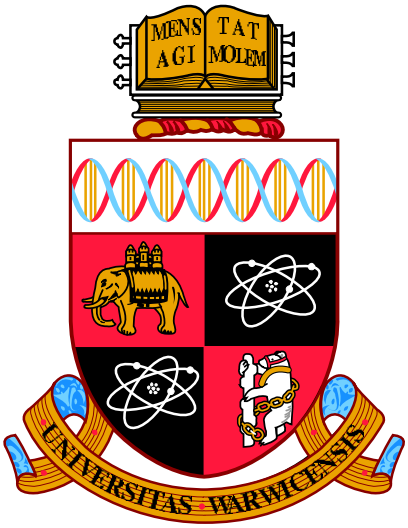


Regulatory networks controlling *ANAC092* expression during different stress responses in *Arabidopsis thaliana*

by

Adam Richard Talbot

Thesis

Submitted to the University of Warwick

for the degree of

Doctor of Philosophy

Systems Biology Doctoral Training Centre

September 2014

THE UNIVERSITY OF WARWICK

Contents

List of Tables

List of Figures

Acknowledgements

I have a number of people I need to thank, without whom this thesis would not have been possible.

Firstly I would like to thank my supervisors, especially Vicky Buchanan-Wollaston who gave me this opportunity. Their support and patience made this project possible. I would also like to thank my advisory committee (Jim Beynon, Andrew Mead and Jose Gutierrez-Marcos) for their suggestions and advice.

I would also like to thank the many people who helped me in the lab or otherwise with this project: Claire Hill, Justyna Prusinska, Peijun Zhang, Emily Breeze, Ana Mendes, Alison Jackson and the rest of the PRESTA group, past or present. To all my friends and colleagues that have made my time here so much more enjoyable: Phil Law, Alex Jironkin, Sarah Harvey, Christine Hicks, Mary Tetlow, Claire Stoker, Jack Grundy, Krzysztof Polanski, Perry Bateman, Jemma Taylor, Max Newbert, David Schafer, Anthony Knight, Mark Walsh, Richard Westbrook and many, many more.

A big thank you to my parents, grandparents and the Curnows who have helped and supported me for so long, even when I stopped being able to explain exactly what it was I was doing at work. Also my girlfriend Harriet for encouraging me when I'm being at my most grumpy. Finally to my uncle Peter, who promised to proof read this for me but never got the chance.

Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by myself except where otherwise stated.

Abstract

Senescence is the final developmental stage of a plant leaf, which occurs as the organ reaches the end of its natural lifecycle. In this process, photosynthetic machinery is broken down and recycled as nutrients for use in the rest of the plant. This allows the recovery of the resources invested in the plant leaves as the plant transitions to a reproductive state.

While a developmentally controlled process that occurs naturally as the organ ages, leaf senescence can also be induced prematurely by environmental stresses. The onset of senescence facilitates survival of the plant as a whole at the cost of leaf tissue. As such, senescence is controlled by regulatory systems that cross link developmental and stress response signals.

One such signalling mechanism is the action of transcription factors. The NAC family transcription factor ANAC092 has been shown to promote the onset and progression of senescence during developmental and stress conditions. Timeseries gene expression data and network inference indicated ANAC092 as being a central hub in the stress response to *Botrytis cinerea* infection.

ANAC092 was shown to be a functional gene during *Botrytis cinerea* infection, promoting the spread of lesions through the onset of a senescence like process. As such, *ANAC092* represents a major cross-link between developmental and pathogenic stress response signals.

A number of transcription factors bound to the *ANAC092* promoter region in a yeast 1-hybrid experiment, including members of the NAC, MYB, ERF and AtHB transcription factor families. Interestingly, members of the same transcription factor family co-localised to regions of the *ANAC092* promoter, suggesting they recognised similar sequences.

Gene expression profiles and network inference were used to generate a model of regulation by these transcription factors. It was predicted certain transcription factors would regulate *ANAC092* expression during specific conditions. In particular, different members of the NAC family of transcription factors appeared to regulate *ANAC092* expression during *Botrytis cinerea* infection or developmental senescence. These predictions were tested using transgenic Arabidopsis.

The NAC transcription factor recognition sequence is known for a limited subset of NAC transcription factors. In an attempt to define the binding motif for a higher proportion of the NACs, a yeast 1-hybrid library of 94 NAC transcription factors was constructed and screened against a range of Arabidopsis promoter regions. Binding motifs were reverse engineered and tested for NAC protein binding.

Abbreviations

3AT	3-Amino-1,2,4-triazole
ABA	Abscisic acid
ABRE	Abscisic acid response element
ACC	1-aminocyclopropane-1-carboxylic acid
AD	Activation domain
ANOVA	Analysis of Variance
At	*Arabidopsis thaliana*
BD	DNA binding domain
bHLH	Basic helix-loop-helix
bp	Base pair
BSA	Bovine serum albumin
bZIP	Basic leucine-zipper protein
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
CATMA	Complete *Arabidopsis thaliana* MicroArrays
CDS	Coding DNA sequence
ChIP	Chromatin immunoprecipitation
Col 0	*Arabidopsis thaliana* Columbia ecotype
DAMP	Damage-associated molecular patterns
DAS	Days after sowing
DB	DNA binding
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
DREB	Dehydration responsive element binding
EMSA	Electrophoretic Mobility Shift Assays
ER	Endoplasmic reticulum
ERF	Ethylene response factor
ESE	Ethylene and Salt Inducible
ET	Ethylene
ETI	Effector triggered immunity
FDR	False discovery rate
GAL4	Yeast transcriptional activator
GFP	Green fluorescent protein
GO	Gene ontology
GRN	Gene regulatory network

HB	Homeobox protein
hCSI	Hierarchical causal structure identification
His	Histidine
HIS3	Yeast histidine biosynthesis reporter gene
Hpa	*Hyaloperonospora arabidopsidis*
HPI	Hours post infection
JA	Jasmonic acid
KO	Knock-out
LB	Lysogeny broth
LD	Long day
Leu	Leucine
LIMMA	Linear Models for Microarray Data
LRR	Leucine rich region
Luc	Luciferase
MAANOVA	Microarray analysis of variance
MAMP	Microbe-associated molecular patterns
MeJA	Methyl jasmonate
MEME	Multiple EM for motif elicitation
miRNA	MicroRNA
mRNA	Messenger RNA
MUG	4-methylumbelliferyl ß-D-glucuronide hydrate
MH-VBSSM	Metropolis variational Bayesian state space model
MRM	Multiple Reaction Monitoring
MYB	Myeloblastoma
NAC	No Apical Meristem, ATAF1, Cup-Shaped Cotyledon
NASC	Nottingham Arabidopsis Stock Centre
ORA	Octadecanoid Responsive
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PRESTA	Plant Responses to Environmental STress in Arabidopsis
PSSM	Position specific scoring matrix
Pst	*Pseudomonas syringae* pv. tomato DC3000
PTI	PAMP triggered immunity
qPCR	Quantitative PCR
RGB	Red/Green/Blue values
RMA	Robust multi-array average
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid

SD	Synthetic dextrose
SDM	Site-directed mutagenesis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repression
SRM	Selective Reaction Monitoring
SRMA	TF SRM assay
T-DNA	Transfer DNA
TAIR	The Arabidopsis information resource
TBP	TATA binding protein
TF	Transcription factor
Trp	Tryptophan
TSS	Transcription start site
UAS	Upstream reporter sequence
UTR	Untranslated region
WT	Wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Y1H	Yeast one-hybrid
Y2H	Yeast two-hybrid
YPDA	Yeast peptone dextrose adenine media

1. Introduction

1.1. Motivation

1.1.1. Feeding an ever increasing population

Despite advances in agriculture meaning fewer people go hungry now then 10 years ago, an estimated 1 in 8 people still do not consume enough nutrients to maintain an active and healthy lifestyle (FAO, 2013), due to a lack of available, affordable food. This problem will be exacerbated by the exponential nature of population growth that is estimated to occur over the next half century. The UN estimates world population will reach 9.5 billion by 2050 (UN, 2014). Feeding this population will require an increase in food production, primarily from greater and more efficient land use. Agriculture already occupies roughly 38% of the terrestrial surface, the largest use of suitable land on the planet (Ramankutty *et al.*, 2008). Expansion of this land will require invasion of ecological reservoirs such as forests within the tropics (Gibbs *et al.*, 2010).

Furthermore, with the increasing population comes an increase in the wealthy population, with an enhanced purchasing power. The new middle classes demand a higher proportion of their diet to be from processed food, meat, dairy and fish, all of which require additional farming at the prototrophic level (Godfray *et al.*, 2010). The increased demands of this expanding population will contribute to the escalation of land use worldwide, thus amplifying the problem.

Agriculture and associated processes are now recognised as one of the major sources of greenhouse gases and thus one of the greatest contributors to climate change, which in turn, will negatively affect the yield of agriculture worldwide (Snyder *et al.*, 2009; Wheeler & von Braun, 2013). Therefore, ensuring agriculture is environmentally sound is critical to ensuring an ever growing population can be fed long into the future.

This means there are three critical challenges facing food security. Firstly, more nutritious food needs to be produced to reduce the number of people classified as hungry. Secondly, food production needs to meet the growing and more affluent population who demand better food. Finally, this needs to be done in a way that is environmentally sustainable. Meeting these three challenges are one of the key targets of plant science.

1.1.2. Increased crop productivity is a key target for plant science

There are two primary strategies for increased food production. Primarily, more land can be reserved for agriculture. Each unit of land that is farmed requires more irrigation, fertiliser, workforce and machinery, all of which contribute to climate change and consume finite resources. Agriculture has already begun to expand dramatically into the tropical forests, which has had a dramatic negative impact on carbon storage and biodiversity. Furthermore, crops that tend to fare well in these environments, such as sugarcane and oil palm, do not tend to contribute to the world calorie or protein output.

The second option is to maximise the yield from current agricultural land, by reducing losses and amplifying production from current methods. As photosynthetic organisms, plants represent the only input to an essentially closed food system. As such, the optimisation and maintenance of plant growth and development is directly linked to the amount and quality of food produced. Therefore, every sub-optimal condition that affects plant growth has a direct measurable impact on food production. Understanding how the plant responds to these sub-optimal conditions may lead to more resilient plants that can maintain productive value despite the poor external environment, which in turn can reduce dependency on chemical treatments and ensure a consistent yield year-on-year.

1.2. Leaf Senescence

1.2.1. Senescence represents the final developmental stage of a leaf organ

Leaves are the primary photosynthetic organ of a plant, supplying the body of the plant with the glucose it needs to grow to maturity and therefore reproduce. As such, maintenance of leaf tissue ensures the plant can harvest the energy required to grow and produce viable offspring. If a leaf can be preserved as an intact and robust organ for longer, the plant can continue to photosynthesise and therefore produce nutrients which will facilitate a healthier, more nutritious plant. Therefore, maintenance of viable, healthy leaves is a key target of plant science and agriculture.

Because the leaf is the primary production unit of the plant, it is abundant in photosynthetic mechanisms, nutrient storage and intracellular components. For the plant, this represents a problem. A significant proportion of macromolecules are locked within leaf photosynthetic machinery, unable to be used to establish the next generation. For example, Rubisco, the CO_2-fixing enzyme, is the largest repository of nitrogen in living plant tissue and some evidence indicates plants use Rubisco as an internal source of nitrogen when demand exceeds the rhizosphere (Makino *et al.*, 1984). Because the leaf is such a large resource of nutrients, the plant has developed an intricate and complex life cycle for the leaf organ which allows the resources to be

reclaimed. Leaf senescence is this final process of programmed recycling that recovers nutrients locked within the leaf through careful, ordered molecular breakdown and remobilisation of intracellular components.

The life cycle of leaf follows a set pattern. During the early phase of leaf development the leaf is a net importer of molecules, consuming nutrients to be used as it grows and expands. This is sometimes referred to as a 'sink', since it is a consumer of nutrients from the plants (Thomas, 2013). Upon reaching maturity, the leaf has developed its photosynthetic machinery and therefore photosynthesis reaches maximum efficiency. During this period, the leaf acts as an exporter of nutrients to the body of the plant, sometimes referred to as a 'source' of carbon molecules. After a period of maximum export rate from the leaf, the plant transitions to a reproductive stage where tissues such as seeds or fruits become packed with nutrients, to allow them to successfully form the new generation. This is sometimes referred as 'grain-filling' and occurs concurrently with leaf senescence. In this stage, photosynthesis and carbon export declines as the leaf stops being an organ of production. Instead, nitrogen and other nutrients begin to be remobilised and exported to the body of the plant (Himelblau & Amasino, 2001; Masclaux-Daubresse *et al.*, 2008; Masclaux-Daubresse & Chardon, 2011). This is accompanied by catabolism of chlorophyll molecules, causing a distinctive colour change from green to yellow in ageing leaves (Ougham *et al.*, 2005; Hortensteiner, 2006). As such, leaf senescence represents the final stage of the lifecycle of a leaf, allowing the plant to reclaim nutrients previously locked within the photosynthetic machinery for the ongoing survival of the plant and its progeny.

It is important to note there are two forms of leaf senescence, referred to as monocarpic and polycarpic senescence. Monocarpic senescence occurs in plants who exhibit semelparity, that is, they contribute all of their resources into a single reproductive cycle and die soon after (Amasino, 2009). Perhaps the best known example of this is the model plant *Arabidopsis thaliana*, which dies after a single reproductive cycle in optimum conditions (Bleecker & Patterson, 1997). In comparison, polycarpic senescence is the senescence exhibited by deciduous trees which go through multiple reproductive cycles. The primary difference is that plants that conduct polycarpic senescence withhold a number of Shoot Apical Meristems (SAMs) during development which can be used to differentiate to form new organs in the next reproductive cycle (Wang *et al.*, 2009a). Therefore, the plant survives beyond leaf senescence and can continue to grow. Although different in many aspects, different members of plant families can be monocarpic and polycarpic, suggesting the underlying mechanisms of senescence are conserved between the two lifestyles. Indeed, Arabidopsis can be converted to a polycarpic species by simultaneous mutation of the *SOC1* and *FUL* genes, albeit with severe developmental defects (Melzer *et al.*, 2008).

1.2.2. Onset and rate of senescence is critical for crop productivity

As a remobilisation of nutrients, the relationship between leaf senescence and crop productivity was suggested a long time ago (Thomas & Stoddart, 1980). In truth, as with all of biology, the link between senescence and crop yield is dependent on many factors and cannot be simplified to a simple statement. However, modulation of senescence has allowed increased yield from crops in some cases (Gregersen *et al.*, 2013). Because 'greenness' is a simple physiological marker, it has been historically selected for by many farmers and breeders in a variety of crops (Thomas & Ougham, 2014). This is known as the 'staygreen' phenotype, characterised by plants that retain a green pigment in their leaves for an extended period (Thomas & Ougham, 2014). Staygreen plants have delayed or reduced chlorophyll degradation and retain their green pigment beyond the normal time periods. Broadly, staygreen varieties are classified into one of two categories. One type are 'cosmetic staygreens', who are impaired in chlorophyll catabolism only and therefore do not lose their green pigment during senescence. These do not exhibit a delay in other senescence mechanisms and therefore show no altered affect on nutrient remobilisation or cell death (Thomas & Howarth, 2000; Balazadeh, 2014). Meanwhile, the other type are 'functional staygreens', which are delayed in the onset or progression of senescence and therefore have physiological effects which may be desirable.

Functional staygreen varieties are plants exhibiting delayed senescence and therefore will have an extended period of photosynthetic activity and may produce a higher yield. If nutrients produced during photosynthesis can be stored after export from the leaf, there will be a net increase in yield with delayed photosynthesis (Gregersen *et al.*, 2013). The relationship between leaf area duration and yield appears to show a strong positive correlation when yield is measured by total biomass. For example, in maize and sorgum, leaf area duration is positively correlated with grain yield (Thomas & Catherine, 1993; Borrell *et al.*, 2000), while in oilseed rape, leaf area duration is positively correlated with dry mass weight of the plant (Hunková *et al.*, 2011). This suggests that the period of time that leaves remain photosynthetically active directly contributes to the mass of the harvested material, therefore, selection for development of lines with delayed senescence is desirable for increasing final yield.

Similarly, if senescence is artificially delayed, yields have been shown to improve. Cytokinins have been known to retard senescence in detached or attached leaves for a long time, (Richmon & Land, 1957; Zwack & Rashotte, 2013), therefore manipulation of the cytokinin signalling pathway is a common method of manipulating the onset of senescence. The *Agrobacterium tumefaciens* gene *IPT* (*ISOPENTENYL TRANSFERASE*) catalyses the rate limiting step in cytokinin biosynthesis and therefore can be used in synthetic constructs to repress senescence. Early experiments using plants constitutively expressing *IPT* showed severe developmental defects due to the elevated concentrations of cytokinin throughout the plant, but later the promoter

of *SAG12* (*SENESCENCE ASSOCIATED GENE 12*), a gene expressed exclusively in senescent tissue was used to express *IPT* in *Nicotiana tabacum*, selectively repressing senescence only in tissue undergoing senescence (Gan & Amasino, 1995). Indeed, ageing tissue in tobacco plants transformed with P_{SAG12}:*IPT* demonstrate spontaneous cell death without any prior senescence occurring (Wingler *et al.*, 2005). The P_{SAG12}:*IPT* tobacco plants showed enhanced yield compared to their wild type equivalents, with a roughly 52% increase in biomass, 40% increase in seed yield and an 84% increase in number of flowers (Gan & Amasino, 1995). This work has been transferred to rice (Liu *et al.*, 2010), lettuce (McCabe *et al.*, 2001), wheat (Sýkorová *et al.*, 2008), Medicago (Calderini *et al.*, 2007), maize (Robson *et al.*, 2004) and cassava (Zhang & Gruissem, 2005; Zhang *et al.*, 2010) amongst others. However, not all of these have resulted in an improvement in yield (reviewed in Gregersen *et al.* 2013).

1.2.3. Senescence and stress responses in plants

1.2.3.1. Senescence is an adaptive process triggered by stress responses

Senescence is a major developmental process, that is specifically triggered in relation to plant age and developmental stage (Lim *et al.*, 2007). However, senescence can also be induced by external factors such as nutrient supply or stress conditions. This is because during stress conditions, it is inefficient to support photosynthetic machinery, therefore the plant induces leaf senescence as a remobilisation mechanism, thus moving nutrients to the seeds and allowing them to propagate the new generation.

Genes which are known to increase in expression during senescence, known as senescence associated genes (SAGs), also increase during many stress treatments such as dehydration (Weaver *et al.*, 1998; Munné-Bosch & Alegre, 2004), salt-stress (Ghanem *et al.*, 2008), dark-treatment (Zhou *et al.*, 2011) and oxidative stress (Navabpour *et al.*, 2003; Zimmermann & Zentgraf, 2005). This indicates that a plant can utilise premature senescence as a component of the stress response mechanism. As such, many aspects of senescence forms part of the adaptive response to sub-optimal conditions in addition to being the final developmental stage of a leaf.

1.3. The Molecular Process of Senescence

1.3.1. Use of Arabidopsis in studying senescence

Arabidopsis thaliana is the model plant species, due to its relatively short life cycle, small genome and the ease of manipulation. As such, it is probably the most studied organism for any aspect of plant biology, including senescence. The molecular events underpinning senescence onset and regulation have been systematically analysed and determined using various techniques in Arabidopsis, although they are by no-means

complete. What follows is a brief review of molecular events that occur in a senescing leaf in Arabidopsis, although other species will be referred to where they are appropriate.

1.3.2. Regulation of leaf senescence

Senescence acts as both a developmental signal and a response to external stimuli. In normal, unstressed Arabidopsis, a leaf will begin to senesce following a specific period of time after emergence (dependent on environmental conditions), a number which is relatively fixed per leaf, meaning older leaves of the same plant will senesce before younger ones. However, external stresses such as high-salinity, mechanical damage, low-light and pathogen attack can prematurely induce senescence, thus utilising the senescence process as a mechanism in the response. Having said this, a leaf will only senesce once it has reached a certain age, regardless of stress conditions (Grbic & Bleecker, 1995; Weaver *et al.*, 1998; Jing *et al.*, 2002, 2005). Clearly, there must be an integration of developmental signals and stress-response signals that precisely regulates the induction of senescence.

Senescence is not just passive decay of an ageing leaf, it is an active process of recycling, initiated by the plant to ensure the plant remains a viable, fit organism as it reaches the end of its life cycle and begins to reproduce. As an active process, senescence involves the increased transcription of a large number of genes, such as those related to degradation and recycling, alongside the reduction in expression of genes relating to photosynthesis and leaf development (Breeze *et al.*, 2011). Premature or poorly timed gene expression levels could lead to loss of cell viability prior to maximum remobilisation, leading to a low recovery of nutrients from the organ (Hortensteiner, 2002). As such, the senescence process is tightly controlled through levels of hormones and regulatory proteins. The coordinated temporal expression of many hundreds of genes is what drives the process of senescence onwards.

1.3.3. Hormonal Regulation of Senescence

Plant hormones are small molecules that occur at very low concentrations and act as signals for a diverse array of plant processes, including growth, development, reproduction and stress response. Each phytohormone currently identified has a wide range of functions and in many instances, act in an antagonistic or synergistic manner with each other. The studies on hormone signalling in plants have expanded dramatically into an enormous and complicated topic covering many aspects of plant biology. The following is a very brief introduction to the role of phytohormones in senescence and stress. A more comprehensive review was recently published by Jibran *et al.* (2013).

1.3.3.1. Ethylene (ET):

Ethylene is a gaseous hormone composed of two carbon molecules double bonded, most widely known to be responsible ripening of banana fruit. Ethylene signalling is involved in cell division, cell elongation, senescence, abiotic stress response and biotic stress response. Ethylene appears to positively regulate senescence. Ethylene accumulates over time in leaves, while exogenous application of ethylene triggers senescence (Grbic & Bleecker, 1995), but only in mature leaves (Jing *et al.*, 2005), implying that ethylene is a critical signal in induction of senescence once the leaf is 'senescence competent', but not before. Ageing leaves of ethylene insensitive mutants such as *ein2 (ethylene insensitive 2)* and *etr1 (ethylene receptor 1)* show delayed senescence compared to wild type equivalents (Grbic & Bleecker, 1995; Oh *et al.*, 1997), while at a transcriptional level, genes relating to cell wall degradation are downregulated in *ein2* during developmental senescence (Buchanan-Wollaston *et al.*, 2005), suggesting ethylene signalling directly contributes to the dismantling of cell walls during senescence.

Ethylene has a number of other functions in a mature leaf. Ethylene insensitive mutants are more susceptible to the necrotrophic fungus *Botrytis cinerea* (Thomma *et al.*, 1999) and salt-stress (Cao *et al.*, 2007; Lei *et al.*, 2011), suggesting ethylene contributes positively to stress response. In contrast, the same ethylene insensitive mutant is resistant to the hemibiotroph *Pst (Pseudomonas syringae* pv. tomato; Lorenzo *et al.*, 2003), possibly indicating ethylene only contributes to necrotrophic pathogen responses.

1.3.3.2. Jasmonates (JA):

Jasmonates and jasmonic acid (JA) are lipid-based hormones derived from the chloroplast fatty acid alpha-linolenic acid (Creelman & Mullet, 1995). Like ethylene, they regulate an enormous range of functions including fertility, flowering and stress response. The relationship between senescence and jasmonate signalling appears to be complex, although in general it contributes to senescence in a positive manner (He *et al.*, 2002). Jasmonate levels are four-fold higher in senescing leaves than green ones (He *et al.*, 2002) and 60-fold higher in 10-week old leaves than 6-week old leaves (Seltmann *et al.*, 2010b). JA synthesis genes are increased in expression during senescence (Breeze *et al.*, 2011), suggesting senescing tissue actively produces jasmonic acid. In addition, treatment with methyl jasmonate results in increased expression of senescence associated genes (Jung *et al.*, 2007). A jasmonate signalling mutant, *coi1,* shows diminished expression of cell wall metabolism genes during senescence, in a manner similar to ethylene (Buchanan-Wollaston *et al.*, 2005). Indeed, in this experiment, expression of a number of genes was diminished in both *ein2* and *coi1*, suggesting they regulate similar mechanisms. Unlike ethylene, Arabidopsis mutants that are defective in jasmonate synthesis or signalling do not show delayed develop-

mental senescence (He *et al.*, 2002; Schommer *et al.*, 2008), although *coi1* did show delayed chlorophyll loss during dark-treatment (Castillo & León, 2008). Seltmann *et al.* (2010a) proposed that although jasmonate accumulates in senescing tissue, senescence is not mediated through the jasmonate signaling pathway. Instead, they propose that the increase in jasmonate levels is generated by the metabolism of cell membranes that occurs during senescence, which releases jasmonate as an intermediary molecule. Therefore jasmonates and jasmonate signalling may not be directly related to senescence, but may be associated with the senescence process.

Outside of senescence, jasmonate signalling is more commonly associated with response to necrotrophic pathogen and wounding response, where it contributes positively to the response to necrotrophic pathogens, but antagonistically to the salicylic acid mediated response to biotrophic pathogens (Glazebrook, 2005). The role of jasmonate in abiotic stresses is less clear and has been studied far less. Jasmonic acid has been implicated in positively contributing to osmotic stress in barley, tomato, citrus and chickpea (Lehmann *et al.*, 1995; Abdala *et al.*, 2003; Arbona & Gómez-Cadenas, 2008; De Domenico *et al.*, 2012). In rice, pre-treatment with JA greatly enhances their tolerance to cold stress (Lee *et al.*, 1997), while cold stress responses are diminished in Arabidopsis mutants deficient in JA signalling (Shi *et al.*, 2011). In general, it seems jasmonate contributes positively to the response to abiotic stresses.

1.3.3.3. Abscisic Acid (ABA):

Abscisic acid is primarily associated with growth, seed dormancy, germination and osmotic stresses, but it also has a complex set of functions in biotic stresses (Nambara *et al.*, 2010; Lee & Luan, 2012). Externally applied ABA appears to promote senescence and ABA content increases with leaf age (Lee & Luan, 2012). This is accompanied by dramatic increase in the expression of genes related to ABA synthesis during senescence (Breeze *et al.*, 2011). Senescing leaves appear to lose considerable amounts of water, despite the increased concentrations of ABA which should close stomata and prevent transpiration. Instead, ABA signalling induces expression of the gene *SAG113* (*SENESCENCE ASSOCIATED GENE 113*), which reopens stomata (Zhang & Gan, 2012). Overexpression of *SAG113* complements the delayed senescence mutant *atnap* (*NAC-LIKE, ACTIVATED BY AP3/PI*), suggesting a negative feedback loop for SAG113 related senescence involving ABA, although the precise role this plays is unclear (Zhang *et al.*, 2012a). Taken together, these results suggest that ABA promotes senescence, however the presence of negative feedback loops and roles in osmotic stress response seems to complicate analysis of the precise function.

In addition to closing stomata in response to osmotic stress, abscisic acid regulates the mechanism by which stomata close in response to pathogens, which can be used as a point of entry for pathogens such as *Pst* (Melotto *et al.*, 2006). However, once *Pst* has breached the surface of the leaf, it appears to trigger the abscisic acid

signalling pathway to suppress JA, ET and SA signalling and enhance susceptibility (de Torres-Zabala *et al.*, 2007). Indeed, the role of ABA in response to pathogens appears complex and based on a multitude of factors, including the nature of the pathogen, age of the plant and timescale of the infection (reviewed in Asselbergh *et al.* 2008).

1.3.3.4. Salicylic acid (SA):

Like other phytohormones, salicylic acid has an enormous range of functions, including seed germination, seedling establishment, fruit ripening, flowering, abiotic stresses and pathogen response (Vlot *et al.*, 2009). Salicylic acid appears to positively contribute to senescence. Mutants in salicylic acid signalling such as Arabidopsis lines expressing *NahG* or deficient in the *PAD4* gene show delayed senescence compared to their wild type equivalents (Morris *et al.*, 2000), suggesting salicylic acid signalling is critical for induction of senescence. Two thirds of the SA biosynthesis genes are upregulated during senescence (van der Graaff *et al.*, 2006) and SA accumulates in senescing leaves (Breeze *et al.*, 2011). Plants expressing *NahG*, which degrades SA, show reduced expression in a number of genes during senescence, including *SAG12,* (Morris *et al.*, 2000; Buchanan-Wollaston *et al.*, 2005). Notably, these genes are different to the senescence related genes dependent on JA or ET signalling and appear to be specific to developmental senescence, as opposed to dark-induced or cell-suspension senescence (Buchanan-Wollaston *et al.*, 2005).

Salicylic acid is perhaps best known for its role in promoting response to biotrophic pathogens. Biotrophic pathogens aim to maintain cell viability in plants and appropriate nutrients from living tissue (Glazebrook, 2005). In response, salicylic acid promotes cell death through the hypersensitive response and other defence mechanisms. This induces a number of responses, including autophagy and cell death (Yoshimoto *et al.*, 2009). It has been proposed that salicylic acid does not directly regulate senescence, but does influence the abundance of regulators such as reactive oxygen species and other hormones which in turn influence senescence (Jibran *et al.*, 2013).

Outside of stress response, salicylic acid appears to inhibit seed germination, photosynthesis, respiration and growth, but induce flowering (Rivas-San Vicente & Plasencia, 2011). This may suggest salicylic acid is involved in the transition between developmental stages as the plant matures.

1.3.3.5. Cytokinin (CK):

Cytokinins are urea based chemicals with a variety of functions, but are often associated with leaf senescence. Unlike, ET, JA, SA and ABA, cytokinins appear to suppress senescence and in some cases reverse the process. External application of cytokinin causes delayed senescence in detached leaves and intact tobacco plants

(Osborne, 1962; Singh *et al.*, 1992), while gain-of-function mutations in the cytokinin signalling receptor kinase *ahk3* exhibit delayed senescence (Kim *et al.*, 2006). Senescence tissue specific expression of Agrobacterium *IPT*, which catalyses the rate limiting step in cytokinin synthesis, dramatically represses senescence in tobacco (Gan & Amasino, 1995; Wingler, 1998), rice (Liu *et al.*, 2010), lettuce (McCabe *et al.*, 2001), wheat (Sýkorová *et al.*, 2008), medicago (Calderini *et al.*, 2007), maize (Robson *et al.*, 2004) and cassava (Zhang & Gruissem, 2005; Zhang *et al.*, 2010). This suggests cytokinin-mediated repression of senescence is ubiquitous throughout the plant kingdom.

Cytokinin signalling has been implicated in positively regulating response to salt stress and drought, reinforcing the cross-link between osmotic stress and senescence once more (Rivero *et al.*, 2007, 2010; Merewitz *et al.*, 2011, 2012). However, cytokinins have also been observed to promote a negative response to osmotic stress. Treatment of leaves with cytokinin increases stomatal apertures and transpiration rates, thus making plants more likely to lose water (Pospíšilová & Baťková, 2004), while overexpressors of a cytokinin degradation enzyme also show enhanced drought tolerance (Werner *et al.*, 2010; Nishiyama *et al.*, 2011). Cytokinin also has a number of roles in plant development, including inhibiting root growth (Davies & Zhang, 1991). In Werner *et al.* (2010), plants with constitutive cytokinin degradation exhibited increased root growth, which may account for the enhanced tolerance to low water observed.

1.3.3.6. Hormone cross-talk:

It is apparent that plant hormones affect an enormous number of stress response and developmental pathways, so it is logical that some hormone signalling pathways act with similar temporal and spatial patterns. Many hormones have been identified to act in a synergistic or antagonistic manner with each other, which fine-tunes the signalling pathway and resultant response to each particular condition. The influence of multiple hormones on a single, tissue, developmental stage or stress response is critical in defining the physiological changes resulting from the signalling pathway.

Jasmonic acid and ethylene based signalling appears to be overlapping and synergistic, particularly during necrotrophic pathogen infection. Both accumulate rapidly after infection and induce expression of a set of defense related genes that establish a response (Glazebrook, 2005), while mutants of ethylene or jasmonate signalling show increased susceptibility to necrotrophic pathogens (Thomma *et al.*, 1998, 1999). Exogenous treatment with these hormones induces many defense related genes, but simultaneous application results in much higher expression of these genes (Pré *et al.*, 2008; Zarei *et al.*, 2011). The expression of defence related genes such as *PDF1.2*, *ORA59* and *ERF1* requires both jasmonic acid and ethylene based signalling (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003; Pré *et al.*, 2008). In a comparison of *ein2*

and *coi1* during *Botrytis cinerea* infection, both displayed diminished expression of a highly overlapping set of genes (AbuQamar *et al.*, 2006).

The JA/ET synergistic pathway is opposed by the salicylic acid signalling pathway. SA signalling is primarily used in response to biotrophic pathogens, where it is favourable for the plant to induce cell death and suppress responses to necrotrophic pathogens (Jones & Dangl, 2006). As such, salicylic acid rapidly increases in response to certain pathogens and acts to suppress JA/ET signalling and induce pathogenesis related (PR; Spoel *et al.*, 2007). Mutants of salicylic acid signalling show opposing phenotypes to jasmonic acid signalling mutants during pathogen infection (Thomma *et al.*, 1998). Salicylic acid signalling represses JA-induced expression of the *PDF1.2* gene through the expression of the TGA2 interacting protein *GLUTAREDOXIN 480* (*GRX480*; Ndamukong *et al.*, 2007) and *WRKY70* transcription factor (Li *et al.*, 2004, 2006). Interestingly, *WRKY70* is also a positive regulator in leaf senescence, mutants of which exhibit accelerated senescence and repressed expression of both SA and JA/ET defense genes (Ülker *et al.*, 2007), suggesting WRKY70 may be a key regulator of both hormone, stress and senescence related signalling pathways.

There are many other examples of hormonal cross-talk, extending through nearly all developmental stages and/or stress responses. For example during seed germination, ethylene signalling appears to antagonise ABA signalling, as can be shown by hypersensitivity of the ethylene insensitive mutant *ein2* to ABA treatment. However, ethylene signalling seems to be crucial for activation of a number of ABA signalling pathways such as expression of *RD29A* and response to salt stresses (Ghassemian *et al.*, 2000; Wang *et al.*, 2007). As has been previously mentioned, ABA signalling also sometimes appears to suppress both JA/ET and SA signalling pathways to facilitate infection by *Pst* (de Torres-Zabala *et al.*, 2007), while in the ABA biosynthesis mutants *aba1* and *aba2* JA/ET responsive genes are upregulated, suggesting ABA has a negative effect on their expression (Anderson, 2009).

The net result from these high complex layers of interaction is that Arabidopsis stress response and senescence are finely tuned to respond to hormonal changes, through complex interactions between many signalling pathways.

1.3.4. Whole transcriptome changes during leaf senescence

The onset and progress of senescence is accompanied by large changes in the plant transcriptome. Many hundreds of genes increase or decrease in expression level to facilitate the cellular changes that occur during senescence (Buchanan-Wollaston *et al.*, 2005; Breeze *et al.*, 2011). These large scale transcription changes need to be carefully controlled to ensure they occur in the correct spatial and temporal patterns. While there are many methods of regulation, control of transcription is arguably the most important since it is the first step to producing an active molecule, i.e., without transcription no functional product can be produced.

Since the process of senescence involves large scale transcriptome changes in a precise and defined order, the process of transcription can be monitored through whole-genome expression changes (Breeze *et al.*, 2011). In leaf 7 of Arabidopsis Col 0, grown in 16 hour day conditions, the major switch from a mature plant organ to a senescing leaf occurs at approximately 29 to 33 days after sowing (DAS, Breeze *et al.*, 2011). At around this time point, there is a clear and discernible decrease in expression of genes relating to photosynthesis and cell maintenance and a concurrent increase in expression of genes relating to hormone biosynthesis, degradation and transport (Breeze *et al.*, 2011). However, the temporal transcriptome changes can be explored further, showing distinct phases of gene expression over the course of a senescence in a leaf.

1.3.4.1. Decrease in expression of photosynthetic machinery related genes

The process of senescence can begin prior to leaves reaching full expansion. In Arabidopsis grown in 16 hour days, visible symptoms of senescence appear approximately 35 days after sowing, however, decrease in the expression of translation machinery related genes occurs at approximately 19 DAS (Breeze *et al.*, 2011). This includes expression of genes involved in amino acid biosynthesis, tRNA aminoacylation and ribosomal protein synthesis. This indicates that retardation of the ribosomal expansion is occurring, causing *de novo* protein synthesis to decrease as the leaf reaches full maturity.

At around 23 days after sowing, genes involved in redirecting the tetrapyrrole synthesis pathway to chlorophyll biosynthesis are decreased in expression, suggesting synthesis of chlorophyll halts. Similarly, the expression of enzymes involved in the carotenoid synthesis pathway (*LUTEIN DEFICIENT 1*, *2* and *5*) decreases, thus the production of carotenoid for the light harvesting complex II structure decreases alongside chlorophyll biosynthesis. This suggests the continuous production of photosynthetic components halts at around 23 days after sowing, which leads to a decline in photosynthesis shortly after.

Concurrently with the decline in expression of genes relating to photosynthesis, expression of genes relating to regulatory pathways increases. Expression of JA and ABA biosynthesis increases at 23 DAS which is shortly followed by an increase in JA and ABA levels at approximately 25 DAS, alongside expression of JA and ABA responsive genes (Breeze *et al.*, 2011). Similarly, at this point a number of transcription factors from the NAC, WRKY, NFY and Zinc-finger protein families are increased in expression, to modulate the downstream transcriptome changes. The increase in expression genes relating to hormone biosynthesis and transcription factor is brief and appears to reduce shortly after, suggesting there is a 'burst' of signalling, which triggers the ensuing molecular changes.

Shortly after these initial changes in gene expression, a second wave of transcrip-

tional change occurs. At approximately 25 DAS, expression of genes relating to the carbon fixation decreases, including two members of the Rubisco small subunit. This reduction correlates with a drop in total protein content of the leaf, suggesting photosynthetic activity reduces and halts.

Consistent with this, over the next 5 - 10 days expression of many genes relating to photosynthesis declines, including *LIGHT HARVESTING COMPLEX BINDING PROTEINS* (*LHCB*), which coordinate the photosystems of plant chloroplasts (Jansson, 1994) and the transcription factor *GOLDEN2-LIKE* (*GLK2*), which promotes expression of photosynthesis apparatus (Waters *et al.*, 2008). The decrease in expression of these genes appears to occur gradually over the course of senescence and some expression is detectable late into the process, perhaps indicating that some photosynthesis needs to occur to provide sufficient energy for metabolism and remobilisation.

1.3.4.2. Genes increased in expression during leaf senescence

Like the decrease in expression of photosynthetic genes, expression of genes related to degradation and recycling begin before the leaf has fully expanded. Expression of autophagy related genes are high at 21 DAS, indicating autophagic mechanisms may be active prior to the onset of senescence (Breeze *et al.*, 2011). Autophagy, or 'self-eating', is a form of internal recycling conserved across all eukaryotic organisms (Hughes & Rusten, 2007), involving the transport of organelles and/or cytoplasm into the vacuole for degradation (Avila-Ospina *et al.*, 2014). The prevailing theory on autophagy is that it exists to clear unwanted cell components and maintains homeostasis. However, it differs from other degradatory mechanisms in that it is more involved with degradation of long-lived proteins and organelles, as opposed to the rapid breakdown of short lived molecules. Autophagy activity appears to increase during senescence and nutrient deprivation or dark-treatment (Hanaoka *et al.*, 2002; Doelling *et al.*, 2002; Xiong *et al.*, 2005), suggesting it may have a role in clearing and recycling molecules during stress.

If autophagy was a component of senescence, it may be expected that autophagy mutants would exhibit delayed senescence. Instead, it has been repeatedly observed that autophagy mutants show accelerated senescence (Hanaoka *et al.*, 2002; Doelling *et al.*, 2002; Xiong *et al.*, 2005; Phillips *et al.*, 2008). This possibly indicates that autophagy is involved in the clearance of damaged organelles and oxidative species, thus maintaining homeostasis and allowing the cell to continue. In the absence of autophagy, the cell loses viability prematurely compared to the wild type equivalent, therefore senescence is initiated early and uses other mechanisms to clear proteins.

By 27 DAS, expression of metabolism related genes begins to increase. In particular, ß-carotene hydroxylases and the carotenoid cleavage enzymes *CAROTENOID CLEAVAGE DIOXYGENASE 7* and *8* are increased in expression, presumably to

catabolise carotenoid compounds in chloroplasts (Biswal, 1995). At a similar time, expression of two metacaspases, *METACASPASE 1* and *2*, increase. Metacaspases have been implicated in promoting programmed cell death (Coll *et al.*, 2010), therefore they may induce the final step of cell death during senescence. However no functional link between metacaspase expression and senescence has been observed.

Shortly after this, cell wall degradation related enzymes begin to increase in expression levels. Pectinesterases, xylosidases, glucosyl hydrolases, ß-glucosidases, pectate lyases and pectin methylesterase inhibitors are expressed to degrade the cell wall and perhaps release sugars for use in respiration (Lee *et al.*, 2007). Expression of many other catabolic enzymes is then induced, including many carbohydrate-degrading enzymes such as pectinesterases, glycosyl transferases, glucosyl transferases and polygalacturonases. This represents the catabolic phase of senescence, where intracellular components are degraded for recycling.

Alongside the increase in expression of enzymes for carbon based molecule degradation, expression of many protease enzymes increases. Proteolytic activity increases dramatically during senescence (Roberts *et al.*, 2012), while treatment of tobacco with protease inhibitors blocks the degradation of rubisco and retards senescence (Carrion *et al.*, 2013).

In particular, cysteine proteases are frequently associated with senescence, including the senescence specific gene *SAG12* (Grbic, 2003). *SAG12* encodes a cysteine protease that is uniquely expressed towards the final stages of senescence, alongside the yellowing observed as chlorophyll is degraded (Grbic, 2003). However, mutants of *SAG12* do not show any phenotype, suggesting functional redundancy in the proteolytic mechanisms (Otegui *et al.*, 2005).

In addition to *SAG12*, a number of serine- aspartic and metalloproteases are also expressed in senescing tissue and appear to play important roles (Roberts *et al.*, 2012). This is not a disorganised burst of proteolytic activity, instead each protease appears to have highly restricted targets and functions, defined by its subcellular localisation and expression profile. Genomic analysis reveals a number of proteases include plastid targeting peptides (Adam & Clarke, 2002), suggesting a subset of proteases are targeted to the plastids and conduct protein degradation there. In particular, the Zn^{2+} dependent metalloprotease FtsH6 has been implicated in degrading the LHCB3 protein of photosystem II during dark-induced senescence, high-light treatment and *in vitro* (Żelisko *et al.*, 2005). However, mutants deficient in the *FtsH6* gene did not show altered rate of degradation of photosystem II during senescence, suggesting functional redundancy between FtsH metalloproteases (Wagner *et al.*, 2011).

No proteases that are targeted to chloroplasts appear to breakdown Rubisco. Instead, during senescence Rubisco is exported to the chloroplast membrane and expelled by budding to form double membrane bound vacuoles known as Rubisco containing bodies (Chiba *et al.*, 2003; Ishida *et al.*, 2008; Ishida & Yoshimoto, 2008), in

a manner similar to autophagy (Avila-Ospina *et al.*, 2014).

Perhaps the best understood degradation organelle formed during senescence are senescence associated vacuoles (SAVs). These small bodies accumulate in chloroplast containing cells during senescence (Otegui *et al.*, 2005). They are characterised by high proteolytic activity and a pH approximately 0.8 lower then the cytosol, possibly to facilitate protease activity (Otegui *et al.*, 2005). The number of SAVs directly correlates with the rate of chloroplast degradation and the majority of the protease activity in senescing tissue (Martínez *et al.*, 2008; Carrion *et al.*, 2013). Plastid targeted GFP relocates to SAVs during senescence (Otegui *et al.*, 2005), while chlorophyll A and rubisco were identified in SAVs using antibodies and HPLC respectively (Martínez *et al.*, 2008).

1.3.4.3. Control of gene expression through regulation of transcription

The coordinated gene expression of many thousands of genes brings about the appropriate and controlled decrease in photosynthetic maintenance followed by cellular degradation observed in senescing tissue. It is the correct spatial and temporal patterns that allow nutrients to be remobilised efficiently and effectively for continued growth of the plant. Control of this process begins at the transcriptional level.

Within the nucleus, genomic DNA is tightly packed around histone proteins, which are themselves condensed to form nucleosomes. DNA within this tightly coiled bundle is inaccessible to RNA polymerase and therefore genes within this region are unable to be transcribed and their expression is repressed. This level of packaging is dependent on factors such as histone modifications and DNA methylation which are highly adaptive to external stimuli (reviewed in Chinnusamy & Zhu, 2009). Modifications to histone proteins such as acetylation will disrupt the protein-interactions that cause DNA to be condensed, therefore opening the DNA which can then be transcribed. Techniques such as DNase-seq have begun to elucidate the nature of DNA packaging in plant development (Zhang *et al.*, 2012b), but have yet to be applied to plant stress responses.

After DNA is unpacked and exposed to cellular machinery, genes encoded along the newly accessible DNA are then available for transcription to RNA. Transcription of DNA to mRNA in Arabidopsis is primarily catalysed by the enzyme RNA POLYMERASE II, which recognises recognition and assembly of basal transcription apparatus at the core promoter, located on average 70 base pairs upstream of the mRNA coding region of a gene (Smale & Kadonaga, 2003). The best characterised core promoter region is the TATA box, a DNA motif rich in T/As located upstream of the transcription start site in 30-40% of eukaryotic genes, including Arabidopsis (Smale & Kadonaga, 2003; Molina & Grotewold, 2005). The TATA box is recognised by the TATA-recognition protein (TBP) and is usually located 25-35 base pairs upstream from the transcription start site. The binding of the TBP and associated

proteins form the pre-initiation complex which facilitates the binding of RNA Polymerase II, which acts as the first step to transcription.

The formation of the pre-initiation complex is promoted or repressed by auxiliary proteins known as transcription factors (TFs). The binding of one or more transcription factors to the DNA upstream of a coding region facilitates or prevents the formation of the pre-initiation complex and therefore promotes or represses transcription. Transcription factors form transient non-covalent interactions with sequences of DNA 5 - 31bp long (Stewart *et al.*, 2012), known as DNA motifs or recognition sequences. The protein:DNA bonds are inherently weak to allow them be dynamic and respond to stimuli. Indeed, predictions from yeast indicate a higher number of low-specificity DNA-binding motifs are preferable to a lower number of more specific DNA binding motifs (Bilu & Barkai, 2005; Geisel & Gerland, 2011). This is because the higher number of motifs allows modulation of target gene expression through the binding of multiple transcription factors in different combinations. In addition, the higher number of motifs allows greater flexibility in individual recognition sites (Stewart & Plotkin, 2013). Therefore transcription factors offer one of the major sources of dynamic regulation in eukaryotic species.

Arabidopsis contains a disproportionately large number of transcription factors, with approximately 1716 coding sequences representing ~5% of the total number of genes (Jin *et al.*, 2014). Each of these transcription factors will regulate a set of targets, which include, but are not exclusive to, other regulatory proteins. Research into the developmental biology of Arabidopsis has highlighted that small numbers of transcription factors regulate a large number of other transcription factors, therefore acting as a 'master regulator', coregulating large numbers of genes (Meyerowitz, 2002; Kaufmann *et al.*, 2010). The regulatory genes targeted by this master regulator will in turn target 'workhorse' genes, which perform the functional changes in the cell (Kaufmann *et al.*, 2010). Manipulation of the function of these master regulators has dramatic phenotypic effects (Meyerowitz, 2002; Kaufmann *et al.*, 2010).

Multiple transcription factors have been identified as master regulators of senescence. The NAC family transcription factor ANAC092 has been studied in particular detail.

1.3.4.4. ANAC092/ORE1/AtNAC2

The NAC family of transcription factors

The NAC proteins are a large family of plant specific transcription factors characterised by a unique NAC domain (Riechmann *et al.*, 2000; Olsen *et al.*, 2005b). NAC transcription factors were originally identified as two independent proteins, named *NAM* (*NO APICAL MERISTEM*) from Petunia and *CUC2* (*CUP-SHAPED COTYLEDON 2*) from Arabidopsis, so named for the developmental defects induced in mutants of these particular genes (Souer *et al.*, 1996; Aida *et al.*, 1997). Com-

parison between the two coding sequences revealed a conserved domain that was shared with two other proteins in Arabidopsis, subsequently named *ARABIDOPSIS TRANSCRIPTION ACTIVATOR (ATAF) 1* and *2* (Aida *et al.*, 1997). The conserved domain was subsequently named the NAC domain, for *NAM, ATAF* and *CUC2.* Whole genome sequencing and phylogenetic analysis has now revealed this same domain conserved in 110 genes in Arabidopsis (Jensen *et al.*, 2010), as well as in many closely related genes of rice (Nuruzzaman *et al.*, 2010), soybean (Le *et al.*, 2011), potato (Singh *et al.*, 2013), barley (Christiansen *et al.*, 2011), Populus (Hu *et al.*, 2010) and Physchomitrella (Rensing *et al.*, 2008). No NAC proteins have been identified in non-plant eukaryotes. Indeed, NAC proteins have been implicated in the development of specialised water transporting plant tissue, suggesting NAC proteins are partly responsible for adaptation of plants to life on land (Xu *et al.*, 2014), therefore one of the key family of genes separating plants from other eukaryotic species.

The conserved NAC domain originally identified through NAM and CUC2 remains the defining feature of NAC proteins. It is always located at the N-terminus and primarily involved with DNA-binding and dimerisation (Olsen *et al.*, 2005a). The C-terminal region of NAC proteins is more diverse and involved in transcriptional activation and binding to auxiliary proteins (Jensen *et al.*, 2010). The structure of the NAC domain has been analysed in detail through crystallography of ANAC019, which has been used as a model for NAC protein structure (Olsen *et al.*, 2003; Ernst *et al.*, 2004; Welner *et al.*, 2012). The 168 amino-acid NAC domain present in ANAC019 shows a largely beta-sheet structure composed of 5 subdomains, whose sequence does not conform to other identified DNA-binding structures (Ernst *et al.*, 2004). Studies of the crystal structure and in-solution protein revealed that the NAC domain interacts with DNA by inserting the conserved amino acid sequence WKATGTD into the major groove of DNA (Welner *et al.*, 2012). The WRKY transcription factors from plants and GCM transcription factors from Drosophila interact with DNA in a similar manner (Cohen *et al.*, 2003; Yamasaki *et al.*, 2012), but using different sequences (Welner *et al.*, 2012). This could indicate a highly ancestral common ancestor, or alternatively it could be an example of convergent evolution.

The identification of the NAC consensus recognition sequence, i.e. the sequence of DNA that is recognised by all of the NAC proteins has been the subject of much study (Jensen & Skriver, 2014). The first recognition sequence for a NAC protein was identified by yeast 1-hybrid for ANAC019. The protein was identified to recognise a core CGTG sequence in the *EARLY RESPONSIVE TO DEHYDRATION STRESS 1 (ERD1)* promoter region (Tran *et al.*, 2004), which was confirmed to be the recognition sequence using the *in vitro* technique EMSA and SELEX (Olsen *et al.*, 2005a; Bu *et al.*, 2008) . Crystallographic studies indicated ANAC019 dimers bound to two CGTG sequences, on opposite strands and in the opposite direction, separated by 6

base pairs (Welner *et al.*, 2012). In many subsequent studies, the core CGT[G/A] motif or similar has been identified as critical to NAC-DNA interactions (reviewed in Jensen & Skriver 2014), however a limited number have identified alternative sequences. For example, ATAF2 has been identified to bind to a low specificity 35 base pair motif (Wang & Culver, 2012) and CBNAC was shown to bind to a GCTT sequence in the *PR1* promoter region (Kim *et al.*, 2012). A protein-binding microarrray experiment for 12 Arabidopsis NAC proteins revealed differences between many of the NAC recognition sequence for different transcription factors, showing some consistency within particular clades of NAC proteins (Lindemose *et al.*, 2014). As such, the CGT[G/A] appears to be a core sequence of NAC recognition, but this may vary based on the particular NAC gene in question.

The more variable C-terminal domain of NAC proteins has not been identified to have DNA-binding properties, but has been shown to act as a transcriptional activator (Tran *et al.*, 2004; Taoka *et al.*, 2004; Jensen *et al.*, 2010) or repressor (Kim *et al.*, 2007a; Yamaguchi *et al.*, 2010). Ten Arabidopsis NAC transcription factors representing a wide spread of NAC phylogenetics and functions have been analysed for transactivation capability in yeast. In 9 cases, the transactivation capability was encoded by the C-terminal domain. The C-terminal domain of NAC proteins does not conform to a particular tertiary structure, instead it has been shown to remain flexible and form transient structures in some cases, a property known as 'protein intrinsic disorder' (Kjaersgaard *et al.*, 2011). Phylogenetic analysis has indicated that the C-terminal domains of NAC domains are mostly non-conserved and instead contain conserved short motifs (Jensen *et al.*, 2010). Since C-terminal domains provide the transcriptional function of NAC proteins, their low conservation implies that NAC proteins are capable of a wide range of transcriptional activity.

NAC transcription factors were originally shown to regulate developmental signals, where mutants exhibited severe developmental defects (Souer *et al.*, 1996; Aida *et al.*, 1997). Many NAC proteins are known to regulate particular developmental signals such as lateral root formation (Xie *et al.*, 2002; He *et al.*, 2005), organ boundary formation (Weir *et al.*, 2004), secondary wall biosynthesis (Zhong *et al.*, 2006, 2007b), xylem cell speciation (Yamaguchi *et al.*, 2010) and many more. Many NAC proteins function in stress-responsive signals (Puranik *et al.*, 2012; Nakashima *et al.*, 2012). NAC proteins have been shown to be involved in response to dehydration (Fujita *et al.*, 2004; Lu *et al.*, 2006), salt-stress (He *et al.*, 2005; Balazadeh *et al.*, 2010b), pathogen response (Bu *et al.*, 2008; Wu *et al.*, 2009; Wang & Culver, 2012; Kim *et al.*, 2012; Hickman *et al.*, 2013), low oxygen levels (Christianson *et al.*, 2009), oxidative stress (Woo *et al.*, 2004; Balazadeh *et al.*, 2011; Wu *et al.*, 2012b), age-related resistance (Al-Daoud & Cameron, 2011) and heat stress (Shahnejat-Bushehri *et al.*, 2012). A small number, including NAP (Guo & Gan, 2006), JUB1 (Wu *et al.*, 2012a), NAC016 (Kim *et al.*, 2013) and ANAC092 (Oh *et al.*, 1997) have been shown to regulate senescence.

ANAC092 is a major positive promoter of leaf senescence

ANAC092 was originally identified by Oh *et al.* (1997). A population of Arabidopsis were mutagenised using EMS, followed by studying them for a delayed senescence phenotype. Redundant mutations were removed which left four distinct, delayed senescence lines named *ORESARA 1*, *2, 3* and *9* (Oresara meaning long-lived in Korean). *ORESARA2* and *ORESARA3* were determined to be alleles of *EIN2,* suggesting their phenotype was due to disruption of ethylene signalling. Later, *ORE9* was shown to encode an F-box protein (Woo *et al.*, 2001). Map-based cloning of *ORE1* revealed the EMS-mutation was present in the coding region for a novel NAC gene, renamed *AtNAC2* (Kim *et al.*, 2009). Later, a systematic nomenclature system for NAC genes was developed, using a numerical system based on TAIR annotations, i.e., starting at *ANAC001* (*AT1G01010*) and followed by *ANAC002* (*AT1G01720*) and so on (Ooka *et al.*, 2003). This meant *ORE1/AtNAC2* was renamed *ANAC092*.

ANAC092 has a well characterised role as a positive promoter of senescence. *ANAC092* transcript accumulates in leaves during senescence (Kim *et al.*, 2009). Non-functional mutants exhibit a delayed developmental senescence phenotype (Oh *et al.*, 1997; Kim *et al.*, 2009; Du *et al.*, 2014), while overexpressors senesce prematurely compared to wild-type (Balazadeh *et al.*, 2010a; Du *et al.*, 2014) suggesting that ANAC092 directly contributes to the onset of senescence. To demonstrate that *ANAC092* was directly linked to senescence and did not just determine leaf age, Balazadeh *et al.* (2010a) expressed *ANAC092* from an estradiole inducible promoter and these plants displayed visible senescence symptoms 3 days after treatment, which were not observed in DMSO treated transgenic plants or estradiole treated plants expressing an empty vector (Balazadeh *et al.*, 2010a). This suggests that ANAC092 is directly linked to the promotion of senescence.

ANAC092 functions in stress-induced and hormonal senescence

In addition to developmental senescence, ANAC092 (Kim *et al.*, 2009) has a role in response to the multiple stress conditions that induce premature senescence. *ore1* shows enhanced resistance to oxidative stress caused by hydrogen peroxide treatment, characterised by reduced chlorosis and maintenance of photosynthesis for much longer then wild-type (Woo *et al.*, 2004). Tolerance to oxidative stress in *ore1* was accompanied by reduced expression in senescence related genes, suggesting ANAC092/ORE1 promotes senescence in response to oxidative stress (Woo *et al.*, 2004). In a similar manner, salt-stress has been shown to promote senescence in wild-type plants. *ANAC092* expression increases in response to response to salt-stress (He *et al.*, 2005; Balazadeh *et al.*, 2010a), while detached leaves from *ANAC092* mutant plants retain chlorophyll compared to wild-type during salt stress (Balazadeh *et al.*, 2010a), suggesting ANAC092 contributes to salt-induced senescence in Arabidopsis. Cotyledon senescence can be induced by application of nitric-oxide, however this effect is

diminished in *ore1*, suggesting *ANAC092/ORE1* is critical for nitric-oxide mediated senescence induction (Du *et al.*, 2014).

ANAC092 is known to function in hormone mediated senescence. *ANAC092* transcript increases in response to ABA, the ethylene precursor ACC and auxin (He *et al.*, 2005). Interestingly, increase in *ANAC092* transcript following ABA treatment is dependent on ethylene signalling genes *EIN2* and *ETR1,* but not on abscisic acid signalling genes such as *ABI5* and *ABI3* (He *et al.*, 2005), suggesting ANAC092 acts downstream of an ABA-ET signalling confluence. *ore1* plants show delayed senescence following treatment with ABA, ET and MeJA, but did not show developmental phenotypes during seed germination and development under hormone treatment, suggesting ANAC092 functions in hormone mediated senescence, but not hormone signalling (Kim *et al.*, 2011).

ANAC092 regulates a number of senescence related processes in Arabidopsis

A number of experiments have been conducted to determine the direct affect of ANAC092 on gene expression. The C-terminal region of ANAC092 appears to be capable of acting as an activation domain in yeast when ligated to the *GAL4* DNA-binding domain (He *et al.*, 2005; Jensen *et al.*, 2010), suggesting ANAC092 acts as an activator of transcription in Arabidopsis. To determine potential genes transcribed in response to ANAC092 in Arabidopsis, *ANAC092* under the control of an inducible promoter was employed (Balazadeh *et al.*, 2010a,b). Plants were tested for gene expression levels using microarrays 5 hours after estradiole-induction of the *ANAC092* construct (Balazadeh *et al.*, 2010a). 170 genes were identified as significantly increased in expression, while 48 genes were downregulated (Balazadeh *et al.*, 2010a). Of the 170 genes upregulated, 78 (46%) were known to increase in expression during developmental senescence (Buchanan-Wollaston *et al.*, 2005). Later, this experiment was repeated by the same group, but reducing the time from estradiole treatment to sampling to 2 hours to capture the immediate downstream targets of ANAC092 (Matallana-Ramirez *et al.*, 2013). In addition, the results were compared to mesophyll protoplasts transformed with a construct expressing *ANAC092* from the CaMV35S promoter (Matallana-Ramirez *et al.*, 2013). 78 genes were identified as differentially expressed 2 hours after induction of *ANAC092* in the stably transformed Arabidopsis, while 831 genes were differentially expressed in the protoplasts transformed with 35S:*ANAC092*, however only 17 genes were identified as differentially expressed in all three datasets (Matallana-Ramirez *et al.*, 2013). 14 of these increased in expression during age-dependent senescence, suggesting a high correlation between *ANAC092* expression and regulation of a number of senescence associated genes (Matallana-Ramirez *et al.*, 2013). The 17 genes identified as differentially expressed in all datasets included genes related to sugar transport, protein degradation, protein ubiquitination, nucleic acid degradation and protein kinases

(Matallana-Ramirez *et al.*, 2013).

One of the genes highlighted in these studies was *BIFUNCTIONAL NUCLEASE 1 (BFN1)*, a nuclease involved in degradation of DNA during leaf and stem senescence (Pérez-Amador *et al.*, 2000). *BFN1* expression levels were consistently positively correlated with *ANAC092* expression following manipulation of *ANAC092* expression levels, i.e., dramatically upregulated in *ANAC092* overexpressors. Expression patterns from the *BFN1* and *ANAC092* promoter regions were spatially and temporally overlapping, i.e., expression of the reporter gene increases during leaf senescence. However expression of GUS from the *BFN1* promoter during leaf senescence was abolished in *ore1* lines (Matallana-Ramirez *et al.*, 2013). In wild type plants, expression of *BFN1* increased with respect to age, however this effect was abolished in *ore1* lines, suggesting *ANAC092* promotes expression of *BFN1* over time (Matallana-Ramirez *et al.*, 2013). Finally, ANAC092 protein was confirmed to interact with an ACGTA(5n)CTCG motif in the *BFN1* promoter using EMSA, ChIP and protoplast transactivation assays (Matallana-Ramirez *et al.*, 2013).

In addition to having a direct action on rates of transcription via promoter regions, ANAC092 can interfere with the action of other transcription factors. The ANAC092 protein forms a protein:protein interaction with GOLDEN2-LIKE 1 (GLK1) and GOLDEN2-LIKE 2 (GLK2) in a yeast 2-hybrid assay (Rauf *et al.*, 2013). Bimolecular Flourescence Complementation (BiFC) confirmed the interaction between ANAC092 and GLK2 *in planta* (Rauf *et al.*, 2013). *GLK* transcription factors promote chloroplast development and photosynthesis (Waters *et al.*, 2008, 2009), therefore it is appropriate for them to be inactivated during senescence to downregulate the photosynthetic process. ANAC092 binds to GLK1/2 and represses their function, thus allowing photosynthesis to decline and senescence progresses (Rauf *et al.*, 2013). Protoplasts transfected with 35S:*GLK2* and 35S:*ANAC092* do not show GLK2 mediated transcription, while plants simultaneously overexpressing *ANAC092* and *GLK2* show similar expression profiles and phenotypes to plants overexpressing *ANAC092* on its own, suggesting overexpression of *ANAC092* has suppressed the GLK2 mediated changes (Rauf *et al.*, 2013).

ANAC092 is regulated by an ethylene controlled gene-regulatory network

ANAC092 is known to be regulated at a number of levels. *ANAC092* transcript increases in response to treatment with ABA, ethylene and auxin in an ethylene dependent manner (He *et al.*, 2005). Expression of *ANAC092* is increased in response to salt-stress in an ethylene and auxin signalling dependent manner, suggesting *ANAC092* expression during premature senescence requires ethylene and/or auxin signalling pathways (He *et al.*, 2005). In the same study, induction of *ANAC092* expression during salt stress was determined to not be dependent on ABA signalling, despite *ANAC092* transcript accumulating after ABA treatment (He *et al.*, 2005).

This suggests *ANAC092* is downstream of a number of hormone signalling pathways that converge on *ANAC092*.

Expression of *ANAC092* was confirmed to be directly linked to ethylene signalling through EIN2 and EIN3 (Kim *et al.*, 2009; Li *et al.*, 2013; Kim *et al.*, 2014). Age-induced expression of *ANAC092* requires the ethylene signalling protein EIN2 (Kim *et al.*, 2009; Li *et al.*, 2013) and the ethylene responsive transcription factor EIN3 (Li *et al.*, 2013). *ANAC092* transcript increases over time, however this effect is abolished in *ein2* or *ein3 eil1* mutants (*EIL1* is functionally redundant to *EIN3).* Overexpression of *EIN3* caused premature senescence and a significant increase in *ANAC092* transcript levels compared to wild type (Li *et al.*, 2013; Kim *et al.*, 2014). In a time-series ChIP-seq experiment, EIN3 bound to the promoter region of *ANAC092* following ethylene treatment (Chang *et al.*, 2013). This was repeated in plant lines overexpressing flag-tagged EIN3 (Kim *et al.*, 2014).

The miR164 family are known to target six NAC gene transcripts through targeted degradation of a conserved sequence in the coding region for their NAC domains (Sieber *et al.*, 2007). Typically for plant micro RNAs, there are three redundant forms of the same miRNA (A, B and C) which target the same NAC genes, although with slightly different expression patterns (Sieber *et al.*, 2007). Many of the NAC genes targeted by *miR164* are involved in organ development (Baker *et al.*, 2005; Peaucelle *et al.*, 2007; Sieber *et al.*, 2007) and boundary formation (Mallory *et al.*, 2004; Laufs *et al.*, 2004; Raman *et al.*, 2008), suggesting *miR164* regulates the spatial arrangement of tissue during development.

ANAC092 is negatively regulated by *miR164*, which helps establish the age-dependent expression pattern. *miR164* is expressed at high levels in young tissue, but begins to decrease in expression levels as the leaf matures and senesces (Kim *et al.*, 2009). This causes *ANAC092* transcript levels to increase over time, concurrently with the decrease in *miR164abc* levels. Overexpression of *miR164B* causes decreased *ANAC092* transcription, while expression of miR164-resistant *ANAC092* restores the wild-type phenotype (Kim *et al.*, 2009). However, *ANAC092* mRNA levels did increase over time in *miR164abc* mutants, which suggested that a redundant mechanism is in place for *ANAC092* expression over time.

The decrease in *miR164abc* expression over time is brought about by EIN2 and EIN3. EIN2 activation causes a decrease in expression of *miR164* over time (Kim *et al.*, 2009), through the transcription factor EIN3 which directly acts on the promoter of *miR164A*, repressing the transcript (Li *et al.*, 2013). In this way, EIN2 promotes a gene regulatory network which controls expression of *ANAC092* in an age-dependent manner. Initially, *ANAC092* is not being actively transcribed and any 'leaky' transcription of *ANAC092* is cleaved by *miR164* directed degradation. EIN2 is activated by ETR in response to ethylene (Ji & Guo, 2013), which activates a signalling cascade which induces expression of *ANAC092*. Activation of EIN2 causes EIN3 to bind to the promoter region of *ANAC092* and *miR164*. EIN3 simultane-

ously suppresses expression of *miR164* and induces transcription of *ANAC092*. The loss of *miR164* and active transcription of *ANAC092* causes *ANAC092* transcript to accumulate which, in turn, induces senescence.

The story with regards to stress induction is not as clear. *miR164* transcript does not decrease during salt-stress (Balazadeh *et al.*, 2010a), despite an increase in *ANAC092* expression during salt stress (He *et al.*, 2005). In addition, *ANAC092* promoter fusions have been used to demonstrate the promoter region of *ANAC092* is active in salt-stress (Balazadeh *et al.*, 2010a), therefore regulation of *ANAC092* must be partly mediated by the promoter region. Plants constitutively expressing *ANAC092* showed premature senescence, but did not show senescence in leaves that had not reached to maturity, possibly because *ANAC092* transcript was degraded in the young leaves (Balazadeh *et al.*, 2010a). The authors suggested that *miR164* exists to 'buffer' *ANAC092* expression and prevent ANAC092 being synthesised early, thus repressing senescence until it is appropriate.

1.4. *Botrytis cinerea* infection and senescence

Senescence is a developmentally induced process, controlled by the maturity of the leaf, but in addition, it is a stress induced process that can be triggered by external factors. One of these external factors that induces senescence is the infection by pathogenic species. Upon infection by a pathogen, a plant will initiate a range of responses, that include, but are not exclusive to, senescence. Since these two processes are linked, the detection and response to pathogens is interwoven with the regulatory elements that control senescence processes. Infection by the necrotrophic fungus *Botrytis cinerea* induces senescence in the area immediately surrounding the lesion site in Arabidopsis (Swartzberg *et al.*, 2008). As such, a brief description of the infection process and response to *Botrytis cinerea* is appropriate.

1.4.1. *Botrytis cinerea* is a classical necrotrophic plant pathogen

A wide variety of pathogens infect Arabidopsis, each with different lifestyles and methods of infection. All pathogens exist with the aim of sequestering nutrients from Arabidopsis, but use different methodologies to extract them. The most well known example of this is the contrast between necrotrophs and biotrophs (Oliver & Ipcho, 2004). Necrotrophs kill cells through toxins or induced cell-death and then feed on the nutrients released, while biotrophs feed on living tissue and therefore aim to maintain cell viability to harvest nutrients (Panstruga, 2003; Glazebrook, 2005). Within these two broad categories are far more divisions, with different pathogens using different strategies to feed off plant tissue.

Botrytis is a fungal necrotroph with a broad host range (van Kan, 2006). Often referred to as grey mould, due to the formation of grey condia on infected tissue,

it is a major crop pathogen causing losses in nearly 200 species (Williamson *et al.*, 2007). As a necrotroph, its primary mode of action is to consume nutrients released from dead plant cells killed using toxins and lytic enzymes. As such, the infectious cycle of Botrytis is relatively straightforward compared to many other pathogens (Schumacher & Tudzynski, 2012).

Conidia germinate on plant tissue before forming appresorial-like structures which penetrate the thick plant cuticle (Williamson *et al.*, 1995; van Kan, 2006). At this point the Botrytis establishes a primary infection site by killing cells in the immediate vicinity. The infection can then enter a latent phase, however this is normally reserved for flowers or developing fruit (Holz *et al.*, 2007). More commonly, after a short lag phase Botrytis infection will lead to a wet lesion and begin to spread. Botrytis excretes a mix of endopolygalacturonases for degradation of plant cell wall pectin and proteases for degradation of plant proteins (Have *et al.*, 1998; Wubben *et al.*, 1999; Kars *et al.*, 2005; Espino *et al.*, 2010), the which provide the primary nutrients for Botrytis growth. Alongside this Botrytis secretes oxalates to acidify the environment to generate the optimum environment for the pathogen degradation enzymes (Manteau *et al.*, 2003; van Kan, 2006).

The degradation of plant cell walls and proteins generates a spreading lesion from the initial infection site which generates the necrotic lesion of a Botrytis infection (Zhang *et al.*, 2014). As such, these enzymes are required for full pathogenicity and establishment of the infection (Have *et al.*, 1998; Kars *et al.*, 2005). Toxins such as botrydial are secreted into the vicinity to kill cells before they can mount an appropriate response (Reino *et al.*, 2004; Fernández-Acero *et al.*, 2007). The concentration of toxins directly correlates with virulence, suggesting they are critical for infection.

Botrytis is often regarded as one of the only 'true necrotrophs', that is it infects a broad host range by a relatively simple mechanism of cell degradation followed by consumption of the nutrients released. As such, it does not subvert the immune system using effector proteins as many biotrophic pathogenic organisms will. Having said this, Botrytis does hijack host machinery for establishment of the full infection. In fact, full pathogenicity of Botrytis requires host participation (van Kan, 2006; Williamson *et al.*, 2007).

Botrytis is known to induce the hypersensitive response in Arabidopsis using an unknown elicitor (Govrin & Levine, 2000; Govrin *et al.*, 2006). The hypersensitive response is a form of programmed cell death triggered in response to pathogen attack to inhibit the spread of biotrophic organisms by preventing access to living cells (Jones & Dangl, 2006). However, Botrytis releases elicitors in the area surrounding the hyphae to promote cell death and therefore lesion size (Govrin *et al.*, 2006).

Similarly, Botrytis produces reactive oxygen species (ROS) at hyphal tips and plasma membrane during infection (Schouten *et al.*, 2002). Histochemical staining revealed that this oxidative burst is composed of hydrogen peroxide, nitrogen per-

oxide and autophagosome-like vesicles at the host-pathogen interface (van Baarlen *et al.*, 2007). Furthermore, the concentration of ROS at the infection site is correlated with pathogenicity (Tiedemann, 1997), suggesting the production of ROS is a positive mechanism for infection by Botrytis. Rapid transient oxidative stress at an infection site is one of the key elements of pathogen response by Arabidopsis (Apel & Hirt, 2004), so it seems odd that Botrytis contributes to the production of ROS. However, Botrytis appears to be immune to the high concentrations of hydrogen peroxide (Temme & Tudzynski, 2009) possibly due to Botrytis producing catalases for clearance of hydrogen peroxide (Schouten *et al.*, 2002). This suggests that Botrytis has co-opted the production of ROS for infection establishment.

Finally, Botrytis can induce premature senescence. Expression of the senescence specific gene *SAG12* increases in leaves infected with Botrytis, suggesting senescence is promoted around the lesion (Swartzberg *et al.*, 2008). Expression of *IPT* from the *SAG12* or *SAG13* promoter in tomato causes a severe delay in senescence, but also confers enhanced resistance to Botrytis infection (Swartzberg *et al.*, 2006), suggesting senescence is critical for establishment of a full infection. As such, it is believed Botrytis exploits the host senescence mechanism to facilitate its own infection.

1.4.2. Recognition of *Botrytis cinerea* by *Arabidopsis thaliana*

Plants have a suite of surface receptors embedded in their extracellular membrane which identify microbial proteins and markers (known as Microbe Associated Molecular Markers, or MAMPS; Boller & Felix, 2009). These receptors are often referred to as plasma membrane (PM)-resident pattern recognition receptors, or PRRs. PRRs contain an extracellular domain which is capable of binding to a particular protein or oligosaccharide unique to microbes. Evolution has driven these to be molecules that are critical for the microbe but not present in plants, such as flagellin or chitin. In addition, a number of these receptors recognise molecules released upon damage to the plant architecture, such as pectin released from plant cell wall degradation, known as Damage Associated Molecular Marker (DAMP).

Upon treatment with the MAMPs or DAMPs, PRRs are activated and the intracellular domain will begin to trigger the response, known as PAMP-triggered immunity (PTI). Many of these surface proteins identified have been Receptor-Like Kinases (RLKs), with an intracellular Ser/Thr kinase domain (Shiu & Bleecker, 2003), which indicates they may have an intracellular enzymatic activity in addition to their extracellular recognition activity. Perhaps the most well known example of this is the flg22 receptor FLAGELLIN-SENSITIVE 2 (FLS2; Gómez-Gómez & Boller, 2000), whose intracellular domain rapidly forms a complex with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) after recognition of the flg22 (Chinchilla *et al.*, 2007; Sun *et al.*, 2013). In turn, the FLS2/BAK1 complex binds to BOTRYTIS INDUCED KINASE 1 (BIK1), who phosphorylate each other and trigger a number of immune

responses such as a MAPK signalling cascade, rapid increase in Ca_{2+}, production of phytoalexins and deposition of callose to reinforce the cell wall and close stomata (Anil *et al.*, 2013).

As a fungus, Botrytis does not utilise flagellin and does not contain flg22. As such, identification of MAMPs for Botrytis has taken some time. The fungal protein chitin is a necessary component of fungal cell walls and therefore largely immutable in evolutionary terms, indicating it was likely to be a MAMP for Botrytis. Eventually the protein CHITIN ELICITOR BINDING PROTEIN (CEBiP) was identified in rice, which was shown to recognise chitin and trigger immune responses such as MAPK activation, reactive oxygen species generation and expression of defence related genes (Miya *et al.*, 2007).

1.4.3. Transcriptional changes during *Botrytis cinerea* infection

Upon detection of a pathogen at the exterior, plant cells will initiate a range of responses to combat the new threat. Defence mechanisms come in many forms, but all require the synthesis of new proteins and therefore pathogenic response is an active process rather then a passive resistance. The activation of defence pathways can be observed by whole genome expression changes (Windram *et al.*, 2012).

Initially, there is a lag phase where very little change in gene expression is observed for approximately 12 hours post infection. This is shortly followed by rapid increase in transcription of genes relating to biosynthesis of ethylene synthesis, particularly *1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) SYNTHASES* (*ACS2* and *ACS6*), which catalyse the rate limiting step in ethylene biosynthesis. Ethylene is one of the critical hormones involved in the response to necrotrophic pathogens, with mutations of ethylene signalling cause enhanced susceptibility to necrotrophs (Thomma *et al.*, 1999). Therefore expression of genes relating to ethylene synthesis helps generate ethylene that is used to trigger the ET-mediated pathogen responses (Thomma *et al.*, 1999; Díaz *et al.*, 2002).

Shortly after this, expression of genes that relate to ethylene response signalling dramatically increase (Windram *et al.*, 2012). This is concurrent with the upregulation of a number of genes relating to jasmonic acid signalling, which acts in concert with ethylene. The JA/ET signalling pathway promotes a significant proportion of the responses to necrotrophic pathogens such as *Botrytis cinerea*, therefore this represents the beginning of the active response to the infection.

At around 14 hours post infection, expression of genes relating to photosynthetic mechanisms such as chlorophyll biosynthesis reduce, alongside an increase in the expression of genes relating to chlorophyll metabolism and degradation. The downregulation of photosynthetic mechanisms has been observed in a number of plant-pathogen interactions. It is suggested to be a mechanism by which the plant reallocates nitrogen compounds for use in the synthesis of defence proteins (Bilgin *et al.*,

2010).

Later, changes in expression of a number of metabolism related genes occur, presumably to clear metabolites that accumulate during Botrytis infection, such as camalexin, flavonoids and glucosinolates (Denby *et al.*, 2004; Kliebenstein & Rowe, 2008). Metabolites such as these are critical to establishing a full defence to *Botrytis cinerea*, therefore rapid synthesis of a sufficient number of enzymes for production of this are critical.

During infection, Arabidopsis reinforces the cell wall to physically restrict the spread of Botrytis (Ellis & Turner, 2001; Ellis *et al.*, 2002). In response to the infection, Arabidopsis induces expression of the cellulase synthase genes *CeSA1* and *CeSA3*, which presumably reinforce the cell wall for enhanced resistance to Botrytis. A mutation of *CeSAV3*, known as *cev1*, shows constitutive activation of the the jasmonic acid and ethylene pathways, thus enhancing the expression of a number of stress responsive genes that confer enhanced resistance to *Botrytis cinerea* infection (Ellis & Turner, 2001; Ellis *et al.*, 2002). This may indicate cell wall modifications share a direct link to phytohormone signalling, although the mechanism behind this is unclear.

Botrytis cinerea produces a number of toxins whose role is to lyse the cell in order for the contents to be released (Amselem *et al.*, 2011). In response, Arabidopsis produces a number of genes that may relate to the catabolism of these toxins, such as glutathione s-transferases (GST), two of which have been implicated in detoxification of synthetic toxins (Dixon *et al.*, 2009).

Finally, there are a number of classical biotic stress response marker genes that are frequently expressed in response to necrotrophic pathogens. Subsequently these genes have often been used as a marker of pathogen response. These include *PLANT DEFENSIN GENE 1.2 (PDF1.2)*, which is rapidly expressed in response to activation of the ethylene and jasmonic acid signalling pathways (Penninckx *et al.*, 1998). *PDF1.2* and other *PDF* genes appear are similar to other eukaryotic defensin genes and have anti-fungal properties (Broekaert *et al.*, 1995; Stotz *et al.*, 2009).

1.5. Analysis of Gene Regulatory Networks

Regulation of gene expression in Arabidopsis is controlled by a large number of processes, incorporating the combined influence of a number of factors. These combine in synergistic and antagonistic ways to ensure correct temporal and spatial expression of genes. The analysis of these factors acting on each other is often conducted as analysis of gene regulatory networks (GRNs). In these networks, each node on the graph represents a single gene, while the edges connecting each node represents the influence of each gene on each other. This helps to elucidate the signalling pathways that control gene expression through a visual representation. Determining the topology and layout of each GRN is critical to determining how the network transmits

signals.

GRNs can be determined using a variety of methods, however, all techniques are aimed at elucidating the edges of a network, that is, the way a gene influences the expression of another gene, or the gene is influenced by other genes. There are two primary strategies to uncovering these edges. The first, is to detect the propensity of a protein to influence a gene through physical interactions. For transcriptional regulation, this is frequently conducted as analysis of direct interaction of proteins on the promoter region of another gene. Another method of determining GRNs is inference, which uses other sources of data to statistically infer the influence of one gene on another through computational analysis.

1.5.1. Physical Methods of GRN analysis

GRNs can be reconstructed through systematic analysis of particular proteins and/or promoter regions for possible sources of regulation. These fall into two broad categories, 'TF-centered', which studies a particular transcription factor for potential downstream targets; and 'gene-centered', which attempts to analyse all potential regulatory elements for a particular gene.

Chromatin immunoprecipitation (ChIP) There are multiple techniques for identification of DNA-protein interactions. Perhaps the most widely known is Chromatin immunoprecipitation (ChIP). In this technique, DNA-binding proteins are purified after chemical cross linking to genomic DNA, normally using formaldehyde. This enriches for target DNA, which can be quantified using qPCR (ChIP-PCR), high throughput sequencing (ChIP-Seq) or microarrays (ChIP-Chip). Although ChIP techniques are often regarded as the 'gold standard' of DNA binding, they come with a number of limitations. The optimum ChIP protocol uses a primary antibody to the protein, the availability of which is limited in plants due to the high degree of homology between plant transcription factors. Even if a primary antibody is available, the low abundance of stress responsive transcription factors often limits what is possible. Both of these limitations can be compensated using transgenic plant lines constitutively expressing native or tagged proteins, however this disrupts the *in vivo* environment and requiring extensive cloning, negating the primary advantages of ChIP techniques.

Protein Binding Arrays Microarrays, normally associated with transcriptome analysis, can be modified for detection of protein-DNA interactions. A large range of oligos bound to chips are used to bind proteins of interest. The protein binding targets can be reconstructed by comparison of known DNA sequences. Similar to ChIP-Seq or ChIP-Chip this can identify transcription factor binding sites in an unbiased manner, but is limited by the limit of sequence size that can be linked to arrays (~10bp). This often restricts protein binding arrays to identification of target DNA motifs, as

opposed to detection of *in vivo* protein-DNA interactions. In addition PBMs require purification of target proteins, although unlike ChIP-based experiments proteins can be purified from bacterial or yeast heterologous expression.

Yeast 1-hybrid An alternative approach uses a gene-centered technique, focusing on a region of DNA and identifying which transcription factors can bind to it. Yeast 1-hybrid is one of the more popular techniques for this, using a region of DNA linked to a reporter gene in yeast. Transcription factors are coexpressed alongside this reporter construct and in the instance where the transcription factor can bind to DNA the reporter gene is expressed and can be detected. This technique has previously been used in plants for a number of transcription factor-DNA interactions (Tran *et al.*, 2007; Chen *et al.*, 2010; Zhu *et al.*, 2010; Hickman *et al.*, 2013; Kim *et al.*, 2014). Because this technique uses a known region of DNA and can identify novel transcription factors, it is often the most appropriate when studying the regulatory elements of a particular gene, as opposed to the role of a particular transcription factor.

Selective Reaction Monitoring Assay (SRMA) Finally, with the development of more sensitive mass spectrometry equipment, new techniques are being developed to detect protein-DNA interactions without antibodies or cloning. This has recently been demonstrated in yeast to determine transcription factors binding to the *FLO11* promoter in affinity chromatography (Mirzaei *et al.*, 2013). The mass spectrometry used to identify the proteins requires a filtering technique called selective reaction monitoring (SRM), sometimes known as multiple reaction monitoring (MRM). This dramatically increases sensitivity and allows quantification of the low abundance transcription factors but does require prior knowledge of target proteins or DNA. With time, it is possible we will see the development of more techniques such as this in the future.

Limitations of Physical GRN Analysis Analysis of gene regulatory networks is never trivial, however molecular techniques such as this offer a significant challenge in terms of time, money and technical expertise. While technology such as cloning systems, robotic instruments and high-throughput platforms have increased the scale of these experiments, they are still limited to studying small numbers of genes at a single time. If we wish to approach a global gene regulatory network, that could be used to identify all the regulatory systems in place in Arabidopsis at any one time, all the Arabidopsis coding regions would need to be studied simultaneously.

1.5.2. Theoretical methods of GRN analysis

Network inference, or *in silico* analysis, is often used to reverse engineer a gene-regulatory network. The premise is that gene regulatory networks can be reverse

engineered from large, undirected data. Data can come in many different forms, but when referring to gene regulatory networks that control gene expression, they are frequently timeseries expression data, that is, expression levels of genes over time. The idea is that if one gene affects the expression of another, then the alteration in expression level of the one gene will be detectable in the expression level of the other, i.e., if a gene that induces the expression of another gene dramatically increases, it stands to reason that the target gene will also change in expression levels. By comparing the timeseries expression data for each gene using computational methods, the global gene regulatory network can be determined.

Technical sources of timeseries expression data

Network inference from these techniques is a powerful tool, made stronger by the availability of whole genome data. DNA microarrays are a common source of whole-genome expression levels (Maskos & Southern, 1992; Schena *et al.*, 1995). Gene expression microarrays are glass slides embedded with a number of DNA molecules at fixed, known locations. These are known as probes, where the sequence of each DNA molecule relates to a coding region of gene or gene fragment. To analyse gene expression levels, mRNA is extracted from biological tissue, before being converted to cDNA and labelled with a dye. The labelled cDNA is hybridised to the probes on the microarray and intensity of the dye at that particular probe is proportional to the amount of cDNA relating to that particular probe. Originally microarrays were limited to small numbers of genes, for example the first microarray for Arabidopsis contained probes representing 45 genes (Schena & Davis, 1992). The availability of complete genomes sequences allows microarrays representing the vast majority of the transcriptome to be analysed, such as the Affymetrix ATH1 chip, which covers 22,555 genes in Arabidopsis (Hennig *et al.*, 2003).

A more recent technology is RNA-seq, which quantifies the level of mRNA present by generating a cDNA library from the mRNA and sequencing this library. The number of times a particular cDNA region is sequenced should directly correlate to the proportion of mRNA present in the original sample (Wang *et al.*, 2009b). RNA-seq offers a number of advantages over microarrays, including higher sensitivity, lower RNA requirements and larger dynamic range (Marioni *et al.*, 2008). They also do not require a reference genome, therefore new transcripts can be detected (Grabherr *et al.*, 2011). However, despite the rapidly decreasing cost of sequencing, RNA-seq still requires complete sequencing of a transcriptome which is prohibitively expensive, particularly when using multiple time points.

The principle behind obtaining timeseries data is relatively straightforward. During the condition that is being studied, many samples are taken over time and analysed for gene expression levels using microarrays, RNA-seq or an alternative technique. By arranging the gene expression level of a single gene over many time points,

you gain a timeseries profile, that is, the expression level of each gene over multiple timepoints. Once this has been obtained, a reverse engineering algorithm is required to infer how this gene expression profile may influence other gene expression profiles.

1.5.2.1. Network inference algorithms

There are many methods of network inference, using a large range of data sources and strategies (reviewed in Bansal *et al.*, 2007 and Penfold & Wild, 2011; reviewed in the context of Arabidopsis abiotic stress response in Swetlana Friedel, 2012). All methods of network inference attempt to determine whether a gene can influence the expression of other genes from their expression profiles, but there is a wide variety of methodologies and algorithms for doing this.

One of the more straightforward methods of inferring a gene regulatory network is to use correlation of gene expression profiles between genes. If the gene expression curve of one gene is very similar to a second gene, there may be a functional link between the two genes. This is likely to be coregulation, i.e. the two genes are controlled by the same mechanisms, which means the gene expression at any given point is similar. Alternatively, the group of coregulated genes could be controlled by a single gene within their number, since the expression profiles match each other.

There are many techniques for correlation of gene expression profiles across large datasets (reviewed in Usadel *et al.* 2009), but all ultimately produce 'clusters' of genes that share a similar gene expression profile over one or multiple timeseries. However, the functional significance of clusters of genes is difficult to determine. While genes may be coregulated, this may be by broad sweeping mechanisms such as hormonal signalling or PTI rather then the action of individual transcription factors. As such, coexpression is often used to infer 'guilt by association', that is suggesting genes that are coexpressed will share biological function, rather then regulatory networks composed of individual genes.

Dynamic bayesian networks (DBNs) offer an alternative that may be more appropriate to a small scale regulatory system. DBNs are forms of directed acyclic graphs, commonly visualised as a network where each node represents a gene, while each directed edge represents the influence a gene has on the the target gene. Bayesian network inference is conducted by finding the optimum acyclic graph that best describes the expression levels of the target, determined from the expression data of the regulators. This is done by generating a range of random graphs, then determining a score for each graph, then by picking the highest score from all inferred graphs using Bayesian statistics (reviewed in Bansal *et al.* 2007). Bayesian networks are limited in that they are unable to model feedback loops or timeseries data. Dynamic Bayesian networks build on Bayesian network methods by incorporating timeseries data and feedback loops that may be biologically important. Instead of representing each gene with a single node, dynamic Bayesian networks represent each gene with

a set of nodes, for each time point (similar to a vertical stack). The acyclic graph at the first time point is then used to assess the directed acyclic graph at the next time point, which is evaluated as before. This allows the network inference to determine feedback loops and incorporate timeseries data (Beal *et al.*, 2005). An added advantage of dynamic Bayesian networks is they can incorporate unobserved or missing data points.

An alternative approach to DBNs and correlation is to use nonlinear dynamical systems (NDS), such as Causal Structure Identification (CSI; Klemm, 2008; Penfold *et al.*, 2012). The principle is that the data changes over time as:

$$X_i = f(X_1, ..., X_n)$$

Where f represents an unknown nonlinear function. This equation can be summarised and used for network inference as an ODE, but it would need to be parameterised. Instead, because we know the parents[1] of the gene, we can determine the influence of f between the parents and the target directly from the data. This means we can identify the nonlinear relationship between the parents of a gene and the gene itself. Since we know the values of X for both the parents and the child gene (from the timeseries data), we identify the nonlinear function f between data for the parents X_p and the child X_i. This can be done by applying a Gaussian process prior to the function f, which 'converts' each value of X_i to a normal distribution about function f of data X_p . The prediction can then be tested using Bayesian statistics. There is significant variation based on the hyperparameters of the Gaussian process, therefore techniques will optimise the hyperparameters using a walking-optimisation technique. In each successive round of optimisation, the prediction can be tested which can be used to estimate a global prediction, i.e. how many times the prediction was determined to be correct in total, known as a marginal probability. Derivatives of this technique have been developed to incorporate multiple datasets or hierarchical gene regulatory network structures, such as hierarchical causal structure identification (Penfold *et al.*, 2012).

These network inference techniques are highly suitable for resolving small scale regulatory networks and estimating the influence each gene will have on another gene, however they are limited by the sheer computational power and technical expertise required to operate them. This is especially true for whole genome network inference, which requires large amounts of computational power to model the large number of differentially expressed genes.

[1] 'parents' referring to the putative regulators of a gene

1.5.3. ANAC092 as a major hub in multiple stress responses

1.5.3.1. The PRESTA project

Plant Responses to Environmental STress in Arabidopsis was a large systems biology project between the Universities of Warwick, Essex and Exeter whose aim was to study transcriptional networks underlying stress responses in Arabidopsis. The premise was to use a systems biology approach whereby global gene expression data during particular stresses would be used to analyse the regulatory network underlying stress responses. The project aimed to study the regulatory network controlling response to 6 particular stress conditions; *Botrytis cinerea* infection, *Pseudomonas syringae* infection, high-light, drought, and senescence. Senescence was studied as two day lengths, long day (16 hour days) and short day (8 hour days).

The methodology was to obtain gene expression timeseries profiles for the majority of genes in Arabidopsis, from which network inference techniques could be used to reverse engineer many facets of the Arabidopsis stress response network. In doing this, the project generated timeseries expression data for a large proportion of the genes in Arabidopsis during multiple stresses, as well as a variety of techniques and methods of analysing said data for biologically relevant processes.

The long-day senescence timeseries data were generated by growing plants in 16 hour day conditions. Leaf 7 was tagged and harvested every day from 19 to 39 days after sowing (DAS). Two leaves were harvested per day, one at 7 hours into the light period and one at 14 hours into the light period to determine the effect of circadian rhythms on gene expression. The two data points were averaged to form a timeseries of 11 data points, one per day from 19 to 39 DAS. This data was published in Breeze *et al.* (2011).

For the *Botrytis cinerea* infection time-course, leaf 7 of four week old Arabidopsis plants were detached and placed on agar prior to infection. *Botrytis cinerea* inoculum were used to infect these detached leaves by placing multiple 10µl spots on each leaf, at a concentration of 10^5spores/ml. Leaves were harvested every 2 hours from initial infection to 48 hours post infection (HPI). This resulted in 24 time points over 2 days post infection. In addition, a mock treatment was included for a comparison. This data was published in Windram *et al.* (2012).

Both senescence and Botrytis datasets were obtained using Complete Arabidopsis Transcriptome MicroArrays (CATMA), covering approximately 30,343 genes (Sclep *et al.*, 2007). RNA was amplified and labelled before being hybridised to the microarray slide in a dye-swapping experimental design detailed in Breeze *et al.* (2011). This meant there was gene expression data determined by microarray for every timepoint during the stress condition. The combination of high genome coverage and high-resolution temporal data led to the availability of a timeseries data available for the majority of Arabidopsis genes under each stress.

Reverse engineering techniques such as those referred to previously were developed

or adapted for use with this high-resolution data to determine biological significance and underlying stress response networks in Arabidopsis (Penfold *et al.*, 2012). Techniques developed have been employed in publications such as Breeze *et al.* (2011), Windram *et al.* (2012), Hickman *et al.* (2013) and Penfold *et al.* (2012).

To supplement the *in silico* derived gene regulatory networks, the PRESTA group also utilised a yeast 1-hybrid technique. Yeast 1-hybrid was employed by the PRESTA group to detect transcription factor:DNA interactions that may be occurring on the promoters of key stress response genes in Arabidopsis. Therefore, it provided empirical data for gene regulatory networks that may be coordinated by transcription factors in Arabidopsis. The gene regulatory networks generated from yeast 1-hybrid data could be compared and contrasted to *in silico* derived networks. Data from yeast 1-hybrid was published in Penfold *et al.* (2012) and Hickman *et al.* (2013).

1.6. Organisation of this thesis

Network inference employed using the PRESTA timeseries data analysed a number of core genes in the stress response network, that were critical for one or more stress responses in Arabidopsis. *ANAC092*, an Arabidopsis transcription factor known to promote senescence was observed to dramatically increase in expression during infection with *Botrytis cinerea* in the PRESTA high-resolution timeseries dataset. In addition, network inference illustrated ANAC092 as a major hub in the core stress response network for both age-induced senescence and response to *Botrytis cinerea* infection. Clearly, regulation of *ANAC092* requires both an age-related signal and a stress-responsive signal that induces expression of *ANAC092* prematurely in response to stress. The aim of this project was to resolve the gene-regulatory network that controls *ANAC092* expression through transcription factors. Chapter 2 is a description of the methods and techniques used in this thesis. In chapter 3, the role of ANAC092 in *Botrytis cinerea* infection is defined through use of Arabidopsis plants overexpressing or deficient in *ANAC092* expression. In chapter 4, transcription factors capable of binding to the *ANAC092* promoter region are identified using high-throughput yeast 1-hybrid and protoplast transactivation systems. In chapter 5, *in vivo* functions of the yeast 1-hybrid interactions are defined using PRESTA timecourse expression data, then tested using transgenic Arabidopsis. In chapter 6, a yeast 1-hybrid library of NAC transcription factors is generated and used to test DNA binding of 92 NAC proteins simultaneously, in an attempt to refine the NAC binding sequence beyond was has previously been determined. Chapter 7 summarises the content and findings of this thesis, while discussing them in the wider context of gene regulatory networks.

2. Methods and Materials

2.1. Plant material & growth conditions

2.1.1. Plant material

All plant lines were acquired from the European Nottingham Arabidopsis Stock Centre (uNASC, http://arabidopsis.info/) or from the original authors. The following T-DNA insert plant lines were used in this thesis:

In addition, a number of plants constitutively expressing a coding sequence were used. These were an *ANAC092* and an *ANAC056* overexpressor, kindly donated by Dr. Jesper Grønlund. For these plants, the coding sequence was expressed from a Cauliflower Mosaic Virus 35S promoter on a pB7GW2.0 plasmid (Karimi *et al.*, 2002) in a Col 0 background. An Arabidopsis plant expressing β-glucuronidase from the 1500bp ANAC092 promoter region (relative to ATG) previously used in Balazadeh *et al.* (2010a) was kindly donated by Dr. Salma Balazadeh.

2.1.2. Plant growth conditions

Arabidopsis seeds were stratified in 0.1% agarose for 72 hours at 4-7°C in total darkness. Stratified seeds were sown by pipetting to pre-watered soil (6:1:1 Levington F2 compost:sand:vermiculite) in 4cm pots (P24, Desch Plant-pak). Pots were covered in cling film to maintain humidity and placed in a growth chamber to germinate. Cling film was removed and plants thinned to one plant per pot after one week. Plants were grown in standardised conditions of 16 hour days, 20°C, 70% relative humidity, 350ppm CO_2 and 100$\mu mol^2.s^{-1}$ light.

2.2. Fungal growth and plant phenotyping

2.2.1. *Botrytis cinerea* growth and harvesting

Botrytis cinerea strain pepper (Denby *et al.*, 2004) were cultured on sterile apricot halves (Del Monte) in petri dishes at 25°C in complete darkness. Botrytis was grown for 2 weeks prior to use. Botrytis spores were harvested in 3ml sterile water in a class II flow cabinet before being filtered through miracloth to remove mycelium. Concentration of spores was adjusted to 2×10^5 spores/ml before being diluted by a 1:1 ratio of sterile grape juice (Tesco, UK) to a final concentration of $1 \times 10^5$for use.

Line	ATG	T-DNA insert	Gene	Source
anac092-1	AT5G39610	SALK-090154	*ANAC092,* AT5G39610	He *et al.*, 2005
anac018-1	AT1G52880	WiscDsLox364F11	*ANAC018*	Kindly donated by Dr. You-wang KIM from DGIST
anac025-1	AT1G61110	SALK-060459	*ANAC025*	NASC
anac056-1	AT3G15510	SALK-137131	*ANAC056*	NASC
anac102-1	AT5G63790	SALK-030702	*ANAC102*	Christianson *et al.*, 2009
myb108-1	AT3G06490	SALK-076395	*MYB108*	Hickman *et al.*, 2013
bos1	AT3G06490	T-DNA insert with constitutive expression	*MYB108*	Mengiste *et al.*, 2003
ese1-1	AT3G23220	SALK-128736	*ESE1*	Zhang *et al.*, 2011
pif7-1	AT5G61720	SALK-062756	*PIF7*	Leivar *et al.*, 2008

Table 2.1: **T-DNA insert lines plant resources** T-DNA plant lines with associated name, gene insert and source

2.2.2. Phenotyping of Arabidopsis plants to *Botrytis cinerea* infection

Leaves 7, 8 and 9 of 28 day old *Arabidopsis thaliana* were detached and placed on 1% w/v agar in 3 propagator trays (10-15 leaves per tray). A single 5µl droplet of *Botrytis cinerea* inoculum was placed on the surface of each leaf. The propagators were sealed using the matching lids before being placed in a Sanyo 970 cabinet that was set to the same conditions as the growth chamber, but with humidity at 90%.

To determine phenotypes, photographs were taken at 48 and 72 hours post infection (hpi) with a 5cm reference for size. Area of lesion size was manually determined using ImageJ (Abràmoff *et al.*, 2004). A Student's t-test was used to calculate a significant difference from Col 0.

2.2.3. Arabidopsis leaf infection with Botrytis for gene expression analysis

For gene expression analysis, leaf 7 was tagged upon emergence using cotton thread. Prior to infection, leaf 7 was detached and placed on 0.1% agar as before, but multiple 5µl droplets of *Botrytis cinerea* inoculum were placed on the leaf surface at 5mm spacing to ensure even infection load depending on leaf size. Where used, mock treatments were performed by treating leaves in the same manner with grape juice diluted with sterile water at a 1:1 ratio. Infected and mock treated leaves were incubated as before. Samples were flash frozen in liquid nitrogen at the appropriate time point before being stored at -80°C prior to RNA extraction.

2.2.4. GUS staining of Botrytis infected Arabidopsis leaves

Arabidopsis plants expressing *GUS* from the *ANAC092* 1500bp promoter region were analysed for GUS expression during *Botrytis cinerea* infection using a GUS stain. Leaf 7 of 28 day old Arabidopsis plants were detached and infected in the same manner as for gene expression analysis. At 24, 48 and 72 hours post infection 3 leaves were removed and placed in 50% glycerol. Leaves were bathed in 90% acetone for 20 minutes to fix tissue. Leaves were then rinsed in sterile water to remove acetone, before being added to GUS stain buffer composed of 50mM NaH_2PO_4 buffer pH 7.2, 0.2% Triton X-100 (Sigma-aldrich), 5mM Ferrocyanide, 5mM Ferricyanide and 2mM X-Gluc (Sigma-aldrich). The leaves were left overnight at 37°C in the dark before being rinsed with increasing concentrations of ethanol; 25%, 50%, 70% and 95%.

2.3. Dark-treatment and senescence phenotyping

2.3.1. Dark-treatment of Arabidopsis plants

3-week old Arabidopsis rosettes were removed from soil and transferred to petri dishes containing sterile water saturated filter paper. Three rosettes were placed in

each petri dish. Petri dishes were stored in complete darkness in growth chambers at standard conditions.

2.3.2. Phenotyping of Arabidopsis plants during dark-treatment

For Col 0, 15 rosettes were analysed while 9 rosettes were analysed for each transgenic line. Photos of petri dishes were taken every day under four 100W tungsten bulbs against a custom background including a white region for colour normalisation (figure 2.1). Colour of white background and leaf 5 were analysed using ImageJ (Abràmoff *et al.*, 2004) at every day using the colour histogram tool. RGB values for leaf 5 of each plant were determined using a custom script by McHattie (2011). The same script also calculated the ratio between red and green intensity, thus providing a measurement of leaf yellowing. Phenotype was determined by a Student's t-test at each time point, whereby 3 consecutive p-values of <0.05 after R:G values were above 0.75 indicated a significant difference in yellowing rates.

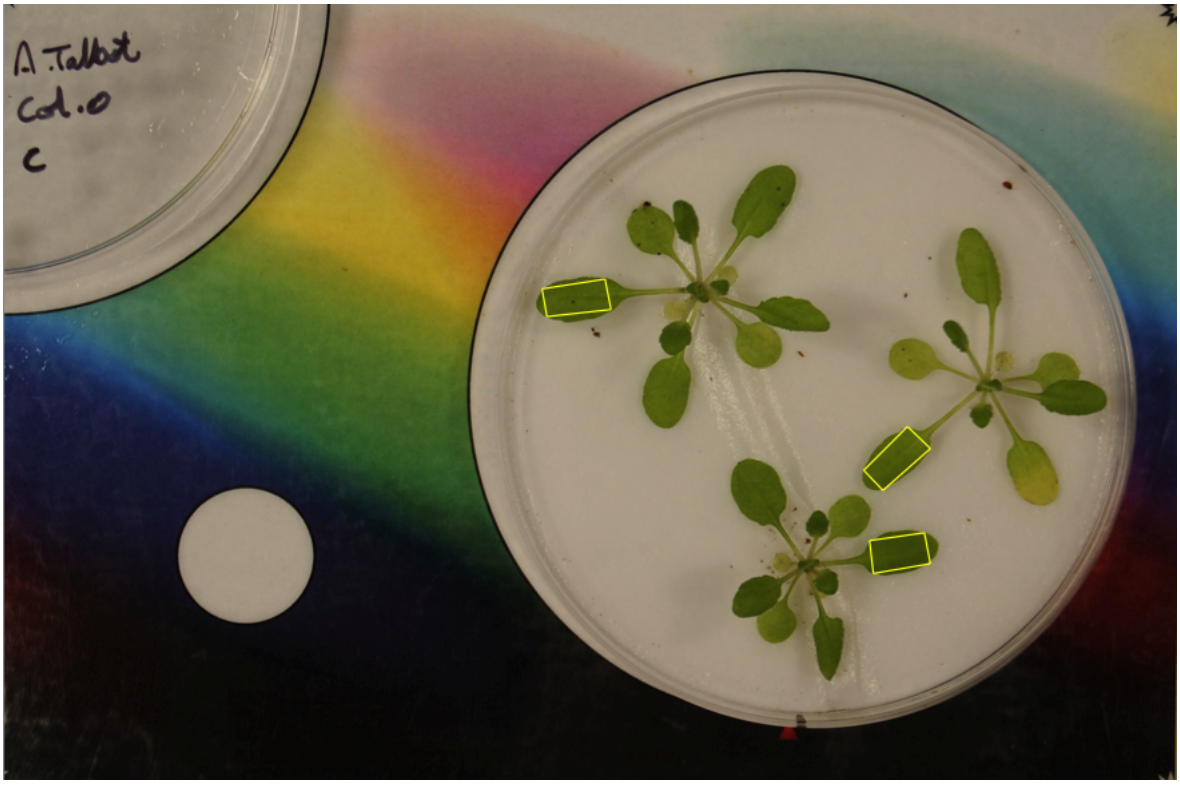


Figure 2.1: **Phenotyping of Arabidopsis during dark-induced senescence** Picture of Col 0 *Arabidopsis thaliana* rosette during dark treatment, 5 days after treatment. Yellow boxes indicate area that R:G ratio is taken from. The background is designed to normalise any white balance errors. In addition, the white circle is used to normalise to pure white and the camera is left on manual white balance (tungsten mode).

2.3.3. Dark-treatment for gene expression analysis

To determine gene expression changes in dark-treated plants, nine rosettes of Col 0 and each line were treated as before. R:G values were measured using the previously described technique on each day. At the point where R:G of Col 0 crossed 0.75, three rosettes from all plant lines were snap frozen in liquid nitrogen prior to storage at -80°C. This was performed on two subsequent days.

2.4. RNA extraction and gene expression analysis

2.4.1. RNA extraction

6mm glass beads were added to frozen tissue before tissue was homogenised using a mixermill with pre-chilled adaptors. Mixermill was used four times at 25w for 30 seconds to grind tissue. 1ml Trizol (Invitrogen) was added to sample before being incubated for 5 minutes at room temperature. 200µl of chloroform (Fisher) was added to each sample, before being vortexed and left at room temperature for 3 minutes. Samples were then centrifuged at 14,000 $\times$ g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh 1.5ml Eppendorf tube, followed by addition of an equal volume of 70% ethanol (Fisher) made using DEPC treated water. This was mixed thoroughly before being added to an RNeasy purification column and purified using manufacturers instructions (QIAgen). Samples were eluted using 50µl of DEPC treated water. Concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo scientific) using a 1.5µl sample.

2.4.2. DNase treatment and cDNA synthesis for qPCR

1000ng of RNA was treated with 1µl of RQ1 DNase (Promega) and 1µl in 10×RQ1 DNase buffer in a total of 10µl DEPC treated water to remove any contaminating genomic DNA (gDNA). RNA with DNase was incubated at 37°C for 10 minutes before addition of 1µl RQ1 stop solution (Promega) and incubation at 65°C for 10 minutes to inactivate any DNase.

cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen). 1µl 50µM oligo$(dT)_{18}$ and 1µl 10mM dNTPs were added to the DNase treated RNA sample, before being incubated at 65°C for 5 minutes to anneal oligos to RNA. 4µl First Strand Buffer, 2µl dithiothreitol (0.1M), 1µl RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen) and 1µl SuperScript II Reverse Transcriptase (Invitrogen) was added to each sample before being incubated at 42°C for 50 minutes, followed 70°C for 15 minutes to inactivate the enzyme. If necessary, cDNA samples were stored at -20°C.

2.4.3. qPCR reaction

qPCR was used to analyse RNA levels of select genes. For each qPCR analysis, primers specific to the target gene were designed to amplify 50-150bp of the coding sequence using NCBI primer blast (primer sequences are in table A.1). Primers designed for amplification of the *PUX1* transcript (AT3G27310) were used as a sample control for each sample.

cDNA samples were diluted by 10 before qPCR analysis (initial concentration of 50ng/µl). 5ng of cDNA was mixed with 5µl of ssoAdvanced SYBR Green Supermix (Bio-Rad) and primers specific for the gene target (200nM), to a total volume of 10µl. Each reaction was performed in triplicate as technical replicates. In addition, for every primer mix a non-template control was included to ensure the reaction mix was not contaminated and a standard curve was included by mixing equal volumes of every sample in the reaction, before serial dilution by 5 multiple times.

qPCR reaction cycle was performed on a CFX384 Touch Real-Time PCR Detection platform (Bio-Rad) in 384-well white skirted BioRad qPCR plates. A 2-step PCR reaction was used, with a pre-cycle 95°C for 3 minutes, followed by 45 cycles of 95°C for 10 seconds, 55°C for 30 seconds. Fluorescence of each well was recorded after each cycle. A post-reaction melt-curve was performed by heating the sample to 95°C for 10 seconds, then performing a temperature gradient increase of 65°C to 95°C at 5 second increments. Fluorescence was measured after each temperature increase. A single melt-curve peak was confirmed visually.

2.4.4. qPCR analysis

qPCR was analysed using the Δ(Ct) method. The cycle number where the fluorescence crossed the threshold (t) was used to quantify the reading from each well, where threshold was set automatically as the point of all fluorescence curves being in the exponential phase. Reaction efficiency was confirmed to be 95-105% using the included BioRad CFX manager software and the standard curve before quantification of the target sample was determined. Gene expression was calculated as the difference between the Ct value of the gene of interest and the Ct of *PUX1* before being calculated to original RNA concentration by assuming a doubling of sample every reaction. Therefore gene expression was quantified thus:

$$\triangle Ct = Ct_{target} - Ct_{PUX1}$$

$$[Relative\,RNA]_{target} = 2^{-\triangle Ct}$$

2.5. Gene Expression Microarray Analysis

2.5.1. CATMA microarray

Gene expression changes for the *ANAC056* overexpressor were determined using a CATMA V4 microarrays (Sclep *et al.*, 2007). Prior to use in microarrays, quality of RNA was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano chip.

2.5.1.1. RNA amplification using MessageAmp^{tm} II aRNA Amplification Kit

Equal amounts of RNA were pooled from each biological replicate, before 11µl was amplified using the MessageAmp^{tm} II aRNA kit (Ambion Biosystems) according to manufacturers instructions, with a single round of amplification and an incubation time of 14 hours. Quality of amplified RNA (aRNA) was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 NanoChip. Concentration of aRNA was measured using a Nanodrop ND-1000 (Thermo scientific).

2.5.1.2. Reverse transcription and dye-labeling

5µg of amplified RNA was reverse transcribed to cDNA using 1.5µg random nonamers (Invitrogen) and 20U RNase OUT (Invitrogen) and DEPC treated water to a final volume of 10.5µl. The solution was incubated at 70°C for 10 minutes, before addition of 4µl First Strand Buffer (Invitrogen), 2µl 0.1M dithiothreitol, 1µl of dNTPs, 1µl of SuperScript II Reverse Transcriptase (Invitrogen) and 1.5µl of 25nM Cy3- or Cy5-dCTP (GE healthcare). Reaction proceeded for 2.5 hours at 42°C. Reactions were conducted using both dyes for both samples, where each sample was labelled with both Cy3 and Cy5 independently. 10µl of 2M MOPS buffer was added to the samples before being purified using QIAquick PCR purification kit (QIAgen) according to manufacturers instructions. Samples were eluted in 30µl of elution buffer.

Labelled cDNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo scientific). 40pM of labelled cDNA was mixed in a dye-swapping design, that is, Cy3-*ANAC056* OE with Cy5:Col 0 and Cy3:Col 0 with Cy5:*ANAC056* OE. Samples were concentrated by freeze drying, before being resuspended in 50µl of pre-hybridisation buffer consisting of 10mg/ml BSA (Sigma-Aldrich), 1 × SSC (Invitrogen) and 0.1% (w/v) SDS (Invitrogen). Resuspended, labelled cDNA was denatured at 95°C for 5 minutes before being applied to CATMA V4 slides in duplicate for technical replicates. Arrays were covered with a cover slip (Sigma-Aldrich). cDNA has hybridised to microarray by placing slides in a high humidity environment at 42°C overnight.

After hybridisation arrays were washed for 5 minutes at 42°C using wash solution 1, then 10 minutes at in wash solution 2 and finally 1 minute in wash solution 3.

The final wash step was repeated 4 times. Arrays were then washed in isopropanol and spun dry for 1 minute at 2000 × *g*.

Wash Solution	Composition
1	2 × SSC (Invitrogen), 0.1% (w/v) SDS (Invitrogen)
2	0.1 × SSC (Invitrogen), 0.1% (w/v) SDS (Invitrogen)
3	0.1 × SSC (Invitrogen)

Table 2.2: **Hybridisation buffers for CATMA arrays** Wash Buffers used to hybridise labelled cDNA to CATMA microarray slides

Arrays were scanned using a 428 Affymetrix scanner at wavelengths of 532nm for Cy3 and 635nm for Cy5. Both scans for Cy3 and Cy5 were combined and processed in ImaGene version 8.0 (Biodiscovery) in order to extract background corrected median data values for each spot on each array.

Expression values were obtained for each spot using LimmaGUI (Wettenhall & Smyth, 2004) in R. Data was normalised within-arrays using print tip loess normalisation. Data was normalised between arrays using Quantile normalisation. To determine differentially expressed genes, a linear model was fitted to the data using the least square method before a Benjamini and Hochberg correction was applied to reduce false positives. A p-value of 0.05 was used as a significance cutoff.

2.5.2. Nimblegen microarray

anac092-1 gene expression changes during *Botrytis cinerea* infection and *anac025-1* gene expression changes during *Botrytis cinerea* infection and dark-induced senescence were determined using NimbleGen 12 × 135K *A. thaliana* gene expression microarray.

Prior to use, quality of RNA was confirmed using an Agilent 2100 bioanalyser with an RNA 6000 Nano chip. Equal amounts of RNA were combined from each biological replicate and SPIA (Single Primer Isothermal Amplification) was performed on 50ng of RNA using the Ovation® PICO WTA kit (NuGEN) according to the manufacturers instructions but modified to use half-volumes of all the reagents. Amplified cDNA was purified using a QIAquick PCR purification kit (Qiagen) and eluted using 30µl of sterile water. 4µg of cDNA was labelled with Cy3 using the NimbleGen one-colour DNA labeling kit (Roche NimbleGen). 3.5µg of Cy3-labelled samples were hybridised to NimbleGen Arabidopsis 12 × 135K microarrays overnight at 42°C, before being washed, dried and scanned on a NimbleGen MS200 Microarray scanner. All steps were performed as per manufacturers instructions.

Raw intensity probe data for each probe and probe background were extracted using DEVA software (Roche Nimblegen), before being normalised intra-array and inter-array using ANAIS (Simon & Biot, 2010). Probe intensity was normalised intra-array using RMA background correction and inter-array using quantile nor-

malisation. ANAIS was used to map probes-to-genes using a median polish process.

2.6. Cloning of genomic and coding sequences

2.6.1. Gel electrophoresis

Unless otherwise stated agarose gels for 1-dimensional separation of DNA were composed of 1.5% ultrapure agarose (Invitrogen), 1 × TAE buffer (40mM Tris base, 20mM acetic acid 1mM EDTA, pH8.0) and 1 × GelRed (Biotium). DNA samples were run on the gel at 100V for approximately 45 minutes. Hyperladder I (Bioline) was used as a size marker for all samples >100bp, while hyperladder V (Bioline) was used as a size marker for samples <100bp.

2.6.2. Amplification of promoter regions from genomic DNA

Promoter fragments were amplified from Col 4 genomic DNA using a two-step PCR designed to add gateway recombination sites to genomic fragments. Primers were designed to specifically amplify the promoter fragment of interest, but also include 12 base pairs of the *attB* site in the PCR product (table A.2). This was subsequently amplified using primers specific to the *attB* sequence. KOD high-fidelity polymerase (Merck) was used to reduce error rates during amplification. Promoter regions were amplified from Col 4 genomic DNA using 1μM of promoter specific primer (promoter regions sequences are included in the appendix), 20ng of genomic DNA and KOD hot start master mix polymerase (Merck), as per manufacturers concentrations. PCR program included a pre-cycle of 95°C for 2 minutes, followed by 11 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 68°C for 2 minutes, followed by a post-cycle extension of 68°C for 2 minutes.

Primers specific to the *att*B site (table A.2, final concentration 0.4μM of each), 1 × KOD hot start master mix (Merck) and water to a final volume of 50μl was added to the product of the previous reaction. A 2-cycle PCR reaction was used, using a low annealing temperature to generate product from the limited primer overlap followed by a 'full' PCR cycle to generate final product. PCR reaction mixture was heated to 95°C for 2 minutes, before 5 cycles of 95°C for 15 seconds, 45°C for 15 seconds and 68°C for 2 minutes. This was followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 68°C for 2 minutes. A final extension of 68°C for 5 minutes was included to conclude PCR reactions. Presence of *att*B-PCR product was confirmed visually on a gel before being purified using QIAquick PCR purification kit (QIAgen). Concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo scientific).

2.6.3. Cloning of PCR product to Gateway® compatible vectors

*att*B-PCR product and the pDONR^{tm}Zeo vector (Invitrogen) were mixed at equal amounts (150ng) in TE buffer pH8.0 to a final volume of 4µl before addition of 1µl BP Clonase® II (Invitrogen) to recombine PCR product into the entry vector. Reaction was left at 25°C overnight.

BP reaction was used to transform gold standard DH5α competent *E. coli* cells (Bioline). Competent cells were defrosted on ice for 10 minutes. 1µl of BP reaction product was mixed with 10µl of competent cells and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds, before being incubated on ice for 2 minutes. 250µl of SOC media was added and cells were incubated at 37°C for 2 hours to allow antibiotic resistance to be expressed. Inoculum was then spread on LB media containing Zeocin (25ng/µl, Invitrogen) and left at 37°C overnight.

Bacterial colonies were inoculated in 100µl of sterile water. Colony PCR was performed on 1µl of the inoculated water using Taq polymerase (Invitrogen). Reaction mixture contained 1 × PCR buffer minus $MgCl_2$, 1.5mM $MgCl_2$, 0.2mM dNTPs, 1µM of *att*B1 forward primer (table A.2), 1µM of *att*B1 reverse primer (table A.2) and 0.25U *Taq* DNA polymerase (Invitrogen). Reaction was as per manufacturers instructions, but with an annealing temperature of 55°C and the extension step was for 1 minute. PCR product was confirmed visually using a 1% agarose gel. This indicated the presence of an insert in the *att*P site of pDONR^{tm}Zeo vector, therefore, positive colonies were inoculated in liquid LB media containing Zeocin (25ng/µl, Invitrogen) and incubated at 37°C overnight with 220rpm shaking.

Plasmids were purified using the geneJET plasmid miniprep kit (Thermo-scientific). Purified plasmids were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo-Scientific). To confirm the correct insert, promoter fragments were sequenced using the M13 forward and reverse primers (table A.2). 500ng of plasmid and 2.5µM of sequencing primer were mixed to a volume of 10µl before being sent to GATC Biotech for sequencing.

2.6.3.1. Cloned transcription factors

As part of the PRESTA project, a number of transcription factors had been previously cloned into Gateway® compatible entry vectors. The majority of these transcription factors were from the REGIA project and consisted of the coding sequence of a transcription factor cloned from Arabidopsis cDNA into the pDEST^{tm}22 Gateway compatible destination vector (Paz-Ares & Regia Consortium, 2002). Where necessary, transcription factors were 'reverse cloned' from pDEST^{tm}22 to pDONR^{tm}Zeo for further use. To do this, 150ng of the pDEST^{tm}22 plasmid containing the transcription factor coding sequence and 150ng of empty pDONR^{tm}Zeo were mixed in TE buffer pH8.0 to a final volume of 4µl, before addition of BP Clonase® II and incubation at 25°C overnight. Reaction mix was then used to transform gold standard

DH5α competent *E. coli* cells (Bioline) as before. Cells were subsequently spread on LB media plus Zeocin (25ng/µl, Invitrogen). Colonies were spread on LB media plus Zeocin and LB media plus Kanamycin (50ng/µl, Invitrogen) to confirm singular transformation by pDONR^{tm}Zeo and not pDEST^{tm}22. Double transformants were discarded, while colonies containing only pDONR^{tm}Zeo were inoculated in 5ml liquid LB media plus Zeocin (25ng/µl, Invitrogen) and left to grow at 37°C with 220rpm shaking. Plasmids were purified from overnight cultures using a geneJET plasmid miniprep kit (Thermo-scientific), before being quantified using a Nanodrop ND-1000 spectrophotometer (Thermo-scientific).

2.6.4. LR recombination reactions

Sequences in pDONR^{tm}Zeo were digested and ligated into a destination vector using an LR reaction. Destination vectors used in this thesis are detailed in table 2.3.

150ng of purified entry vector containing the sequence was mixed with 150ng of target destination vector in TE buffer pH8.0, before addition of LR Clonase^{tm} II and incubation at 25°C overnight. Reaction mixture was used to transform gold standard DH5α competent *E. coli* cells (Bioline) and plated onto selective LB media (selection depends on destination vector, table 2.3). Colonies were spread to LB media plus Zeocin and LB media plus destination vector selective media, to ensure the bacteria were not transformed with both the entry and destination vector. Colonies that grew on the correct media were inoculated in liquid selective media and left overnight at 37°C with 220 rpm shaking. Plasmids were purified from cultures using a geneJET plasmid miniprep kit (Thermo-scientific), before being quantified using a Nanodrop ND-1000 spectrophotometer (Thermo-Scientific). To confirm the correct insert, promoter fragments were sequenced using the pDEST^{tm}22 specific forward and reverse primers (Hickman *et al.*, 2013). 500ng of plasmid and 2.5µM of sequencing primer were mixed to a volume of 10µl before being sent to GATC Biotech for sequencing.

2.6.5. Site-directed mutagenesis

Where necessary, sequences were modified using site-directed mutagenesis in the entry vector, followed by recombination to the appropriate destination vector. Specific primers were designed at the mutation site, with the 5' end of the primer deliberately mismatched to induce the mutation. The two primers were designed to face in opposite directions to amplify the whole plasmid. PCR was performed using 1µM of forward and reverse primers (primers in appendix), 1 × KOD Hot Start Master mix and 25ng of entry vector (pDONR^{tm}Zeo containing sequence to be mutated) and water to a final volume of 10µl. Reaction included precycle 95°C for 2 minutes, followed by 35 cycles of 95°C for 15 seconds, 58°C for 15 seconds and 68°C for 2 minutes. A post cycle extension step of 68°C for 2 minutes was included. Methylated DNA was digested by adding 1µl *Dpn1* (New England Biolabs) and incubating

Name	Use	*E. coli* selection (antibiotic)	Yeast selection (auxotrophic requirement)	Reference
pHISLEU2GW	Yeast 1-hybrid promoter fragments	Kanamycin (50ng/µl, Invitrogen)	Leucine	(Çevik *et al.*, 2012)
pDEST^{tm}22	Transcription factor expression and ligation to GAL4-AD in yeast 1-hybrid	Ampicillin (100ng/µl, Invitrogen)	Tryptophan	Invitrogen
pJIT60	Constitutive expression of gene in protoplasts	Ampicillin (100ng/µl, Invitrogen)	N/A	(Guerineau *et al.*, 1992, Volkan Cevik)
GWGUS1	GUS reporter fusion for promoter regions in protoplasts	Ampicillin (100ng/µl, Invitrogen)	N/A	Justyna Prusinska

Table 2.3: **Gateway compatible destination vectors** Destination vectors used in this thesis. Shown is common name, application and selection in *E. coli* and *S. cerevisiaie* (where applicable). GAL4-AD is the activation domain of the *GAL4* gene from yeast.

at 37°C for 1 hour. The reaction was purified using a QIAquick PCR purification kit (Qiagen) and product was eluted using 15µl of water. Phosphate groups were added to the 5' terminus of the DNA by addition of 10U of T4 Kinase, 1 × Forward Reaction Buffer (Invitrogen) and 1.2µM ATP. Reaction was incubated at 37°C for 10 minutes before being terminated by increasing the temperature to 65°C for 10 minutes. Reaction product was purified using a QIAquick PCR purification kit (QIAgen). Purified product was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo scientific). 150-300ng of DNA was ligated using 1U T4 DNA ligase (Invitrogen) with 1 × T4 DNA ligase buffer made up to 10µl with water. Reaction was incubated at 16°C overnight. 2µl of reaction was used to transform 10µl α-Select Chemically Competent DH5α *E. coli* cells (Bioline) as described in section 2.6.3 and spread to LB media plus Zeocin (25ng/µl, Invitrogen). Colonies were left to grow overnight at 37°C, before being inoculated and purified in the same manner as other entry vector constructs (see above).

2.7. Yeast 1-hybrid

2.7.1. Small-scale transformation of *Saccharomyces cerevisiae*

pHISLEU2GW-promoter constructs were used to transform *S. cerevisiae* strain Y187 (Clontech), while pDEST22-transcription factor constructs were used to transform the AH109 strain of *S. cerevisiae* (Clontech). *S. cerevisiae* was grown in YPDA media (20g/l glucose, 20g/l peptone, 10g/l yeast extract, 100mg/l adenine) and incubated at 30°C with 200rpm shaking. 1ml of yeast cells were pelleted by centrifugation at 2000rpm for 5 minutes, before being resuspended in 1ml 0.1M LiAc. This was centrifuged again at 2000rpm for 5 minutes, before being resuspended in 1ml 0.1M LiAc. The cell suspension was incubated at 30°C for 1 hour, before 100µl of cell suspension was added to a PEG:DNA mix composed of 1000-1500µg plasmid DNA, 8µg salmon sperm carrier ssDNA (denatured at 100°C for 10 minutes and snap-cooled, Sigma-Aldrich) and 290µl 50% PEG 3350 (Sigma-Aldrich), preheated to 30°C. The PEG:DNA:Cell mix was incubated at 30°C for 50 minutes, before being heat shocked at 42°C for 15 minutes, then pelleted at 3000rpm for 15 minutes. Cells were resuspended in 200µl sterile water and spread onto selective Synthetic Dropout plates (SD media by Clontech, see table 2.3 for correct selection). Plates were incubated at 30°C for 2-3 days until visible colony formation.

2.7.2. High-throughput transformation of *Saccharomyces cerevisiae*

In order to transform yeast with multiple plasmid constructs simultaneously a high-throughput yeast transformation protocol was adapted from Gietz *et al.* (1992). AH109 yeast were incubated in 10ml YPDA overnight at 30°C, before being sub-cultured to 200ml YPDA and left to grow for a second night at 30°C. The 200ml

culture was pelleted in four 50ml falcon tubes at 2500rpm for 5 minutes before being resuspended in one 5ml volume of sterile water. This was pelleted again at 2500rpm for 5 minutes and resuspended in 5ml of 0.1M LiAc. This was pelleted once more at 2500rpm for 5 minutes, then resuspended in 2ml 0.1M LiAc. Cell suspension was added to a carrier DNA mix composed of 3ml 1M LiAc, 8µg ssDNA (Sigma-Aldrich) and 1ml of sterile water. 1µl of pDEST^{tm}22 encoding transcription factors (500-1000ng/µl) were aliquoted to each well of a 96-well plate in the correct library formation. 50µl of the AH109 cell suspension was added to each well of the plate. The plate was shaken at 200rpm for 2 minutes to thoroughly mix the yeast cells and plasmid DNA, before 100µl of PEG 3350 (Sigma-Aldrich) was added to each well. The transformation mixture was incubated at 42°C for 1 hour with shaking at 220rpm. Cells were pelleted by centrifugation at 2500rpm for 5 minutes, before supernatant was discarded and pellet was resuspended in 14µl of sterile water. 5µl of cell suspension was spotted to SD minus tryptophan before being incubated at 30°C for 2-3 days. After this, colonies were resuspended in 1ml of liquid selective media in a deep-well 96-well plate. The plate was incubated at 30°C overnight, before a 5µl aliquot was spotted to a selective media plate (table 2.3) and incubated at 30°C to confirm correct transformation. The deep-well plate was incubated simultaneously for 2 days at 30°C. Glycerol stocks were made by centrifuging cultures at 2500rpm for 5 minutes, discarding the supernatant then resuspending the pellet in a glycerol stock mixture composed of 100µl of selective media and 100µl of 50% (w/v) glycerol before being snap frozen.

2.7.3. Inoculation of PRESTA transcription factor library

The PRESTA transcription factor library consisted of 1440 cloned Arabidopsis transcription factors in pDEST^{tm}22 from the REGIA collection (Paz-Ares & Regia Consortium, 2002; Castrillo *et al.*, 2011) individually transformed into AH109 yeast (Clontech). Cloned transcription factors from the REGIA project were a kind donation from the REGIA consortium. The 1440 transcription factors were arranged in two 96-well formations with 24 independent transcription factor clones per well of the plate.

For each yeast 1-hybrid experiment, the transcription factor library was inoculated from glycerol stocks to 1ml SD minus tryptophan media in deep-well 96-well plates and incubated at 30°C for 3 days before use.

Transformation and arrangement of the PRESTA transcription factor library was performed by Dr. Claire Hill and Miss Peijun Zhang.

2.7.4. Inoculation of the promoter fragment fusion

Y187 *Saccharomyces cerevisiae* transformed with the promoter fragment cloned into pHISLEU2GW were inoculated from selective plates to 5ml SD minus leucine media

in 50ml falcon tubes. Inoculums were incubated at 28°C overnight before use.

2.7.5. Mating of bait and prey yeast strains

3µl pHISLEU2GW transformed Y187 and pDEST^{tm}22 transformed AH109 yeast were spotted directly on top of each other on YPDA plates and left to mate overnight at 30°C. This is described in Hickman *et al.* (2013).

2.7.6. Replica plating to selective media

The YPDA mating plates were replica plated to SD minus leucine and tryptophan (SD-LW), SD minus leucine, tryptophan and histidine (SD-LWH) and SD minus leucine, tryptophan and histidine plus 3-aminotriazole (3-AT, Sigma-Aldrich) at one of multiple concentrations. 3-AT was used as competitive inhibitor of HIS3 activity. This was done because *HIS3* was expressed from the cloned promoter region at a certain background rate by endogenous yeast factors, therefore 3-AT was used to suppress any background HIS3 activity. Concentrations of 3-AT were initially 1mM, 5mM and 25mM, however these were adjusted depending on the observable levels of background yeast growth.

2.7.7. Identification of positive results

Plates were photographed using a G:Box EF2 (Syngene). Growth on SD minus leucine and tryptophan was confirmed visually, as a positive indicator of mating, i.e., containing both plasmid constructs. Growth on SD minus leucine, tryptophan and histidine above background rates or negative control indicated a positive interaction of transcription factor with promoter DNA, thus recovering from histidine auxotrophy. Any positive results were restreaked to the same selective media and incubated overnight at 30°C overnight. The sequence encoding the transcription factor was amplified by yeast colony PCR using pDEST^{tm}22 specific primers as described in Hickman *et al.* (2013). PCR product was sequenced from pDEST^{tm}22 specific primers 2.5µM by GATC biotech.

2.7.8. Verification of positive results

All transcription factor - DNA interactions detected in yeast 1-hybrid were verified by complete technical replication. Plasmids were retransformed to fresh yeast and the experiment was repeated in an pairwise manner. Each pairwise yeast 1-hybrid experiment was repeated three times using different transformants and overall positive results were determined as two or more positive interactions.

2.8. Protoplast transactivation assay

2.8.1. Cloning of transcription factors and promoter fragments to protoplast compatible vectors

Transcription factors in pDONR^{tm}Zeo were cloned to an *att*R Gateway recombination site adjacent to the constitutive promoter region on a pJIT60 plasmid (Guerineau *et al.*, 1992) using an LR clonase® II as described before. pJIT60 had been modified by insertion of a Gateway® cassette B (Invitrogen) immediately after the double 35S sequence.

Promoter fragments were cloned from pDONR.Zeo to an *att*R Gateway® recombination site at the 5' side of a minimal 35S sequence and β-glucuronidase (GUS) coding sequence on a GWGUS1 plasmid constructed by Dr. Justyna Prusinska. This plasmid initially encoded 4 × GAL4 4 × D1-3 linked to a -46bp 35S and GUS coding sequence (Tiwari *et al.*, 2001), but the 4 × GAL4 4 × D1-3 sequence had been excised using *PstI* (Thermo-Scientific) before the plasmid was blunt-ended using T4 DNA polymerase (Invitrogen). Gateway® cassette B (Invitrogen) was digested using *EcoRV* (Thermo-Scientific) before being ligated using T4 DNA ligase (Invitrogen). Correct orientation was determined using PCR and sequencing from the minimal 35S sequence.

All LR reactions were conducted as previously described, however sequencing was conducted using the M13 forward and reverse primers. After determination of correct insert, cells transformed with the plasmid were inoculated in 100ml of selective LB and incubated overnight at 37°C. Plasmids were purified using a Maxiprep kit (Qiagen). The precipitated DNA was resuspended in 50µl of sterile water before being quantified using a Nanodrop ND-1000 (Thermo-Scientific).

2.8.2. Isolation of mesophyll protoplasts from Arabidopsis leaf tissue

Mesophyll protoplasts were generated using a protocol adapted from (Yoo *et al.*, 2007). Col 0 *Arabidopsis thaliana* were grown at standard condition for 4 weeks. Leaves 5, 6 and 7 of ~24 plants were detached and cut across the leaf into thin (0.5 - 1mm) strips. Strips were placed into 15ml 0.45µm filtered enzyme mixture consisting of 20 mM MES (pH 5.7), 1.5% (wt/vol.) cellulase R10 (Duchefa-biochemie), 0.4% (wt/vol.) macerozyme R10 (Duchefa-biochemie), 0.4 M mannitol and 20 mM KCl pre-warmed to 30°C. Leaf tissue was vacuum infiltrated at 20bar for 30 seconds, then left to digest in the dark for 4-5 hours until protoplasts were release could be confirmed visually. The protoplasts were then diluted with an equal volume of cold W5 buffer, consisting of 2 mM MES pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl and filtered through a 75µm sieve to remove undigested plant tissue. Protoplasts were pelleted at 100 × *g* for 1 minute, before the supernatant was discarded and protoplasts were resuspended in 5ml of W5 buffer. The concentration of protoplasts

was calculated using a haemocytometer before being pelleted at 100 × g for 1 minute, then resuspended in MMG buffer (4 mM MES pH 5.7, 0.4 M mannitol and 15 mM $MgCl_2$) to make a concentration of 10^6 protoplasts/ml.

2.8.3. Protoplast transfection

Plasmid DNA, consisting of 3µg of pJIT60-*LUCIFERASE*, 5µg of pJIT60-*TF* and 10µg of GWGUS1-promoter fragment to a final volume of 10µl, were mixed with 100µl of buffer containing protoplasts. 110µl of PEG-calcium transfection solution, consisting of 40% wt/vol. PEG4000, 0.2 M mannitol and 100 mM $CaCl_2$, was added to each protoplast suspension before being gently mixed by hand. Protoplast suspensions were incubated at room temperature for exactly 10 minutes before 500µl of cold W5 solution was added to each sample to halt transfection. Protoplasts were pelleted by centrifugation at 100 × g for 2 minutes at 4°C before being resuspended in 1ml of WI solution (consisting of 4 mM MES, pH 5.7, 0.5 M mannitol and 20 mM KCl). Protoplast suspension was incubated for 24 hours at room temperature to express transfected genes. All transfections were repeated in triplicate for technical replicates.

2.8.4. Isolation of protoplast proteins

Protoplasts were pelleted by centrifuging at maximum speed for 1 minute at room temperature. Supernatant was discarded and pellets were resuspended in cold 1 × passive lysis buffer (Promega). Protoplast lysates were vortexed three times and kept on ice, before being pelleted by centrifugation at maximum speed for 5 minutes at 4°C. Supernatant containing protoplast protein extract were transferred to a 96-well round bottomed plate stored at -80°C if necessary.

2.8.5. Luciferase assay of protoplast protein extract

A luciferase assay was used as normalisation of transfection between protoplast suspensions. 25µl of luciferase reagent (Promega) and 25µl of protoplast protein lysate were combined in a luctrac 200 white 96-well plate and immediately measured for luminescence using a GENios plate reader with MAGELLAN software (Tecan). GENios was set to perform an orbital shake of 2 seconds, followed by a settle time of 2 seconds, then record luminescence at 550nm following an integration time of 2500ms.

2.8.6. MUG assay of protoplast protein extract

40µl of protein extract was added to 100µl of MUG assay buffer composed of 1mM 4-methylumbelliferyl ß-D-glucuronide hydrate (Sigma), 10mM Tris-HCl pH 8.0, 2mM $MgCl_2$. 20µl was immediately removed and mixed with 180µl of 0.2M Na_2CO_3 as a zero timepoint. The MUG reaction was sealed and incubated at 37°C in the dark for

18 hours before 20µl was removed and mixed with 180µl of 0.2M Na_2CO_3. The MUG assay was quantified by measuring fluorescence at 465nm from excitation of 360nm using the GENios plate reader. Gain was set to 60, with number of flashes at 3 and integration time at 40µs. As with the luciferase assay, the GENios machine conducted an orbital shake of 2 seconds followed by settle time of 2 seconds. Fluorescence was measured three times and an average of three was taken as the final fluorescence measurement per replicate.

2.8.7. Analysis of protoplast transactivation

Protoplast transactivation was defined as the difference between the fluorescence measurement at T18 and T0 of the MUG reaction, over the transfection efficiency defined by the luciferase reading. Therefore the relative protoplast activation level was:

$$Relative\, MUG/LUC\, units = \frac{MUG_{T_{18}} - MUG_{T_0}}{Luc.}$$

2.8.8. High-throughput transfection of protoplasts

In an effort to improve the reliability of a protoplast transformation, a high-throughput variant was adapted from (Wehner *et al.*, 2011). Protoplasts were generated as before, however they were resuspended to a concentration of 5×10^5 in MMG buffer. 2µg of GWGUS1-promoter and 2µg of pJIT60-transcription factor made to a total of 3µl were placed in 16-wells of a deep-well 96-well plate. 30µl of protoplasts (1×10^4) were added to each DNA mixture and mixed gently on a plate mixer. 33µl of PEG-calcium solution was added to each well and cell suspension was mixed thoroughly by shaking. Protoplast suspension was left at room temperature for 20 minutes before addition of 120µl of cold W5 buffer to halt transfection. Protoplasts were pelleted at $100 \times g$ for 2 minutes at 4°C before being resuspended in 100µl of WI solution. 96-well plate was sealed with a gas-permeable membrane and protoplast suspension was incubated in the dark at room temperature for 24 hours.

The protoplasts were pelleted by centrifuging at maximum speed for 1 minute at room temperature. Protoplasts were resuspended in 50µl 1× cold passive lysis buffer (Promega), before being mixed by pipetting. Protoplasts were left on ice for 15 minutes, before being centrifuged at maximum speed for 15 minutes at 4°C. Protoplast protein lysate was analysed for expression of *GUS* from the promoter region by a MUG assay. 20µl of protoplast protein lysate was added to 50µl of MUG assay buffer. 10µl of the reaction was immediately removed and added to 90µl 0.2M Na_2CO_3 to act as a zero timepoint. The MUG reaction was incubated for 18 hours at 37°C in the dark before 10µl was removed and added to 90µl 0.2M Na_2CO_3 . The MUG assay was quantified using fluorescence as before, however final result was determined as T_{18}-T_0, since no transfection control was present.

2.9. Hierarchical Causal Structure Identification (hCSI)

The hierarchical causal structure identification algorithm (hCSI), developed by Chris Penfold (Penfold *et al.*, 2012) was used to infer network topology for each target using PRESTA timeseries data published in Breeze *et al.* (2011) and Windram *et al.* (2012) and yeast 1-hyrid interactions as a constraining hypernetwork. Initial hyperparameters and prior distributions over the hyperparameters for the Gaussian process priors were set as in Penfold *et al.* (2012) and Hickman *et al.* (2013). The maximum number of transcription factors that could bind simultaneously in the algorithm was restricted to four unless the number of yeast 1-hybrid connections in that particular instance was lower. In these situations it was set lower. Five Markov chain Monte Carlo chains were run in parallel, generating 50000 sample network structures with the first 10000 sampled discarded to allow the algorithm to reach equilibrium. The remaining 20000 samples were used to calculate the marginal probability for each connection in the yeast 1-hybrid network.

2.10. *Hyaloperonospora arabidopsidis* (*Hpa*) growth and plant phenotyping

Hpa growth and phenotyping was performed by Lesley Foster as per Tomé *et al.* (2014). *Hpa* isolate Noks1 was maintained on Wassilewskija-eds1*Arabidopsis thaliana* before use. For phenotyping, 7-day old seedlings were infected by spraying a suspension of 5×10^4 conidiosporangia/ml *Hpa* and emergence of asexual sporangiophores was used as a measure of susceptibility at 4 days post treatment. Phenotype was taken as sporangiophore emergence per plant.

2.11. Data analysis/visualisation

2.11.1. Student's t-tests

Unless otherwise stated, all t-tests performed were unpaired assuming unequal variance.

2.11.2. Heatmaps

Heatmaps were generated in R using the heatmap.2 script in the gplots package. Hierarchical clustering was conducted as part of heatmap.2 using default settings.

2.11.3. Overrepresentation of gene ontology terms

Analysis of overrepresented GO terms were performed in the cytoscape plugin BiNGO, using full Gene Ontologies with mapping from TAIR10. GO terms were determined

as overrepresented using a Hypergeometric test with a Benjamini and Hochberg correction (Benjamini & Hochberg, 1995). Significantly overrepresented GO terms were taken as a p-value below 0.05.

3. The role of the transcription factor ANAC092 in Arabidopsis during infection by *Botrytis cinerea*

3.1. Introduction

3.1.1. ANAC092 in senescence and stress response

The initiation of leaf senescence in optimum conditions is controlled by organ age. However, processes such as high salinity, low light levels, drought, pathogen infection and nutrient deficiency induce senescence prematurely through distinct and overlapping signalling pathways. The response to each stress condition appears to involve a set of stress-specific gene expression changes, however over the course of the stress more generic gene expression patterns are seen, including the increased transcription of a number of senescence associated genes (Guo & Gan, 2011). The convergence of gene expression changes indicates there are central regulators of senescence who respond to multiple stress conditions.

3.1.1.1. ANAC092 in salt-stress

Salt-stress in plants has been shown to cause a decline in photosynthetic activity, suggesting an accelerated rate of natural senescence (Dwivedi *et al.*, 1979; Chen *et al.*, 2012). The implication is that during salt stress, senescence is initiated to dismantle photosynthetic machinery. The early onset of senescence during salt stress appears to be a key mechanism in maintaining homeostasis of leaf organs (Allu *et al.*, 2014).

ANAC092, or ORE1 is a key promoter of leaf senescence (Oh *et al.*, 1997). However, it also functions in salt-stress response. During salt-stress, *ANAC092* transcript increases in an ethylene and auxin signalling dependent manner (He *et al.*, 2005). The leaves of *ANAC092* mutants are tolerant to salt-stress, retaining their chlorophyll far longer then the wild type equivalents (Balazadeh *et al.*, 2010a). Constitutive expression of *ANAC092* from a 35S promoter causes enhanced lateral root formation, similar to wild type plants grown at 150mM NaCl, suggesting salt-stress root growth response is constitutively activated (He *et al.*, 2005). Meanwhile, overexpression of *ANAC092* in groundnut increases high-salt and low-water tolerance, suggesting ANAC092 conserves its abiotic stress response function across species (Allu *et al.*, 2014).

An inducible overexpressor construct of *ANAC092* was constructed by Balazadeh *et al.* (2010a) and used to determine putative targets of ANAC092. Five hours post induction of the transgene in Arabidopsis, 170 genes were significantly increased in expression levels, suggesting they are targets of ANAC092 regulation. Of these, 78 were known senescence related genes, while 57 were responsive to long term salt-stress (Balazadeh *et al.*, 2010b). 36 of the 170 differentially expressed genes were both senescence associated genes and salt-stress responsive, suggesting they represented a salt-triggered regulon controlled by ANAC092 (Balazadeh *et al.*, 2010b). This implies that ANAC092 cross-links the senescence and salt-stress by regulating a distinct set of genes during both conditions.

3.1.1.2. ANAC092 in oxidative stress

Reactive oxygen species (ROS) such as H_2O_2 are harmful chemicals produced as a byproduct of respiration, which need to be cleared from the plant cells through scavenging enzymes and anti-oxidants. However, reactive oxygen species can overcome these defences and induce oxidative stress, which stimulates a number of responses including senescence (Apel & Hirt, 2004). However, ROS perform an alternative function as signalling molecules, inducing responses to abiotic and biotic stresses (Baxter *et al.*, 2014). ROS concentrations increase during senescence, which implies they may act as a positive regulator of senescence processes (Zimmermann & Zentgraf, 2005). Furthermore, transcriptome changes in Arabidopsis during senescence, salt-stress and oxidative stress are highly overlapping suggesting all three conditions promote overlapping gene expression changes (Allu *et al.*, 2014).

In a similar manner to salt-stress, mutants of *ANAC092* appear tolerant to H_2O_2 treatment through maintenance of photosynthetic tissue (Woo *et al.*, 2004). This appears to be due to a failure to induce expression of senescence associated genes, suggesting ANAC092 conserves its role in promoting senescence during oxidative stress. Furthermore, it appears many of the members of the salt-triggered regulon of ANAC092 identified in Balazadeh *et al.* (2010b) are increased in expression during H_2O_2 treatment, suggesting a conserved function across multiple stresses.

3.1.1.3. ANAC092 in age-related resistance

The phenomenon of age-related resistance (ARR) is a process by which mature plants become more resistant to particular pathogens, particularly *Pseudomonas syringae* pv tomato DC3000 (*Pst*; Kus *et al.*, 2002). In Arabidopsis, ARR is triggered by the transition to flowering, rather than by age of the plant (Rusterucci *et al.*, 2005; Develey-Rivière & Galiana, 2007). Originally, it was suggested ANAC092 contributes to establishment of ARR, because *ANAC092* mutants show a normal level of susceptibility to *Pst* at young ages but 6-week old *ANAC092* mutants are more susceptible then Col 0 (Carviel *et al.*, 2009). However, this was later clarified to be a function

of plant age rather then susceptibility. *ANAC092* mutants show a delay in flowering, which correlates with the onset of ARR. Therefore, plants were tested for *Pst* susceptibility at 7 weeks post germination (Al-Daoud & Cameron, 2011). *ANAC092* mutants showed enhanced resistance to *Pst* at 7 weeks, suggesting that while ARR is delayed in *ANAC092* mutants, when it is activated it is enhanced compared to wild type. To corroborate this, plants constitutively expressing *ANAC092* were tested for flowering transition and susceptibility to *Pst*. 35S:*ANAC092* plants displayed accelerated flowering compared to wild type and showed enhanced susceptibility to *Pst*, showing a partial ARR defect. Therefore, in wild type plants ANAC092 contributes positively to flowering and the onset of ARR but negatively to the process of ARR.

3.1.2. Expression of *ANAC092* in PRESTA timeseries data

The PRESTA timeseries data can indicate previously unknown functions for a gene. If a gene is significantly increased in expression levels during a treatment, it implies that the gene is responsive to those conditions. This may also indicate that the gene plays a role in establishment of a response to that stress condition.

In the PRESTA timeseries data, *ANAC092* increases in expression during developmental senescence, in line with a senescence promoting transcription factor (figure 3.1A, Kim *et al.*, 2009). In addition, *ANAC092* transcript increases during infection with *Pseudomonas syringae* (figure 3.1B), consistent with results observed in Carviel *et al.* (2009). Interestingly, *ANAC092* transcript accumulated more with the virulent strain of *P. syringae* pv. tomato (*Pst*) DC3000 than in an avirulent mutant deficient in suppression of the hypersensitive response (Debener *et al.*, 1991), suggesting *ANAC092* expression correlates with the pathogenicity of the bacteria.

ANAC092 was also strongly expressed in response to *Botrytis cinerea* infection (figure 3.1C). Expression of *ANAC092* increased dramatically at 22 hours post infection and remained consistently higher then the mock treatment until the end of the timeseries, suggesting *ANAC092* expression is highly responsive to infection by Botrytis. Senescence is known to occur in the vicinity of Botrytis lesions in infected Arabidopsis leaves (Swartzberg *et al.*, 2008), which may occur through activation of ANAC092.

3.1.3. Network inference implies ANAC092 is central to a number of stress response gene regulatory networks

In an attempt to determine the central regulators that control stress response in Arabidopsis, the PRESTA group focused on identification of central hub genes in the gene regulatory network. 'Hub' genes are members of a gene regulatory network who are disproportionately highly connected compared to other genes. Because they are so highly connected, activation of these hub genes triggers a major switch from one biological process to another (Kaufmann *et al.*, 2010). In the context of stress

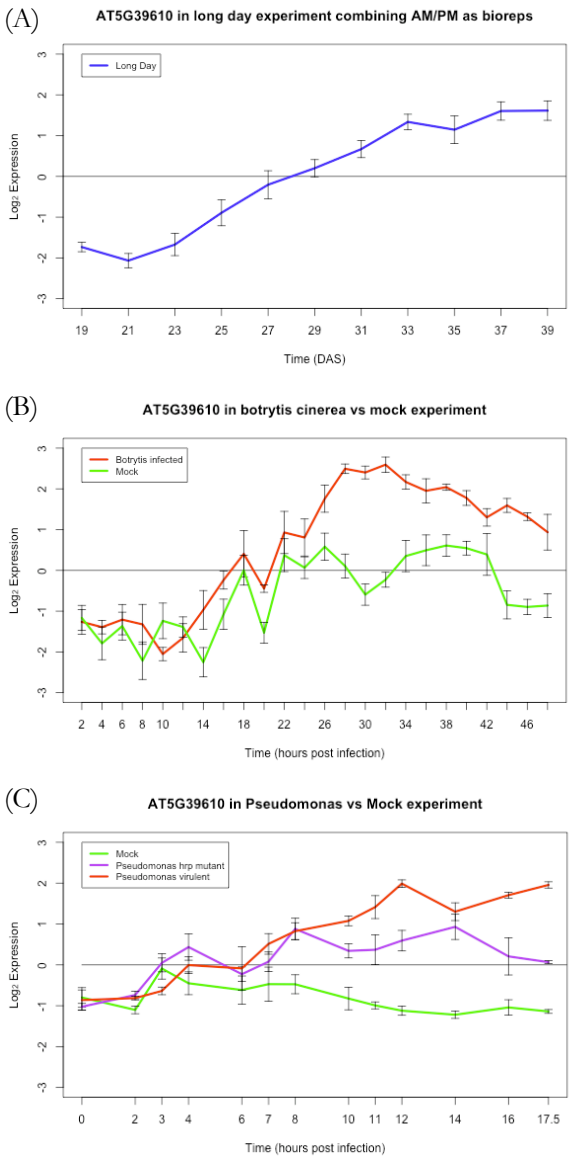


Figure 3.1: **Expression of ANAC092 during senescence and biotic stress** Expression of *ANAC092* during (A) age-induced senescence, (B) *Botrytis cinerea* and (C) *Pseudomonas syringae* infection as determined by the PRESTA high-resolution timecourse microarray. All expression values displayed as Log_2expression with a mean of 0. Figure made in GeneViewer (Stuart McHattie, http://www.wsbc.warwick.ac.uk/stuartmchattie/Senescence/GeneViewer.html)

response, this switch would be from development and growth to response and survival (Huot *et al.*, 2014). As such, identification of major hub genes in the stress response network is key to analysing the core stress response.

The PRESTA project used a network inference technique with the timeseries data to determine the topology of the stress response networks for senescence, Pseudomonas infection and Botrytis infection. The Metropolis-Hastings Variational Bayesian Space State Modelling algorithm (Beal *et al.*, 2005) was used to infer connections

between members of the gene regulatory network in an attempt to define the most connected members, who were suggested to be the central hub genes in the core response network.

Consistent with the role of ANAC092 being a major promoter of leaf senescence, ANAC092 was predicted to be a major hub in the network inferred using senescence timeseries data. Interestingly, ANAC092 was also predicted to be a major hub in the network inferred from Botrytis infection timeseries data, suggesting ANAC092 is a central regulator of response to Botrytis. Therefore, it was hypothesised ANAC092 played a critical role in response to *Botrytis cinerea* infection.

3.1.4. Aims

The aim of this chapter was to analyse the functional role of ANAC092 during *Botrytis cinerea* infection.

3.2. Results

3.2.1. *ANAC092* mutants show an altered phenotype to biotic stresses

3.2.1.1. Expression of *ANAC092* is reduced in *anac092-1* during *Botrytis cinerea* infection

If ANAC092 is a transcription factor involved biotic stress response signalling, disruption of *ANAC092* expression may induce an altered phenotype in Arabidopsis during biotic stress. A T-DNA insertion mutant of *ANAC092* (SALK-090154C, figure 3.2) was retrieved from the Nottingham Arabidopsis Stock Centre (NASC, http://nasc.life.nott.ac.uk/). This line had been previously used to show null expression of *ANAC092* in (He *et al.*, 2005). In this work, *anac092-1* plants exhibited enhanced tolerance to high salinity conditions, suggesting *ANAC092* promoted response to salt-stress. It was decided to test this line for a phenotype during *Botrytis cinerea* infection.

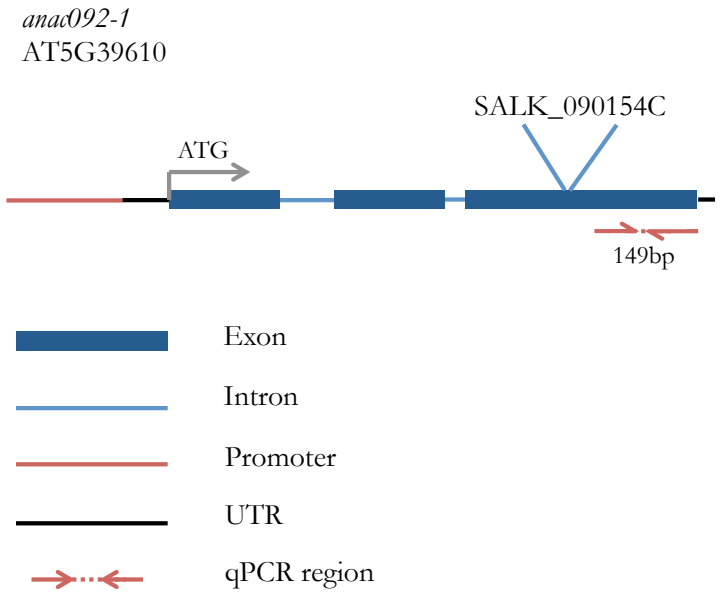


Figure 3.2: **Diagram of coding region of *ANAC092* for *anac092-1*** Diagram of *ANAC092* coding region on genome of Arabidopsis, showing SALK-090154 T-DNA insert.

To confirm that *ANAC092* had reduced expression in this mutant during *Botrytis cinerea* infection, *ANAC092* expression was tested by qPCR in *anac092-1* during *Botrytis cinerea* infection at 28 hours post infection (figure 3.3). Expression of *ANAC092* was significantly reduced in *anac092-1* during Botrytis infection (figure 3.3), therefore *anac092-1* is functional knock-out of *ANAC092*.

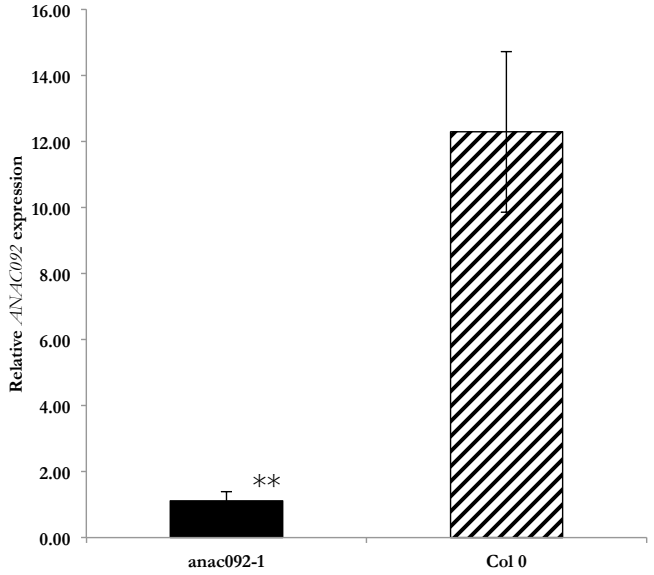


Figure 3.3: **Expression of *ANAC092* in *anac092-1* during *Botrytis cinerea* infection** *ANAC092* expression as determined by qPCR relative to *PUX1* at 28 hours post infection (hpi) with *Botrytis cinerea*. Error bars represent SEM (N=3), ** indicates $p < 0.01$ in a Student's t-test

3.2.1.2. *anac092-1* shows an enhanced resistance phenotype to *Botrytis cinerea* infection

The *anac092* mutant was tested for a phenotype during *Botrytis cinerea* infection. *anac092-1* infected leaves showed a significantly reduced lesion size compared to Col 0 at 72 and 60 hours post infection (figure 3.4). Therefore the lesion size was larger in the wild type plant compared to the mutant. This suggests that ANAC092 is a negative regulator of response to *Botrytis cinerea*.

3.2.1.3. An *ANAC092* overexpressor shows increased susceptibility to *Botrytis cinerea*

Arabidopsis constitutively expressing *ANAC092* was kindly donated by Dr. Jesper Grønlund. An overexpressor of *ANAC092* should demonstrate an opposing phenotype to *anac092-1*, therefore it was expected that the overexpressor would show an increased lesion size compared to Col 0. The 35S:*ANAC092* line was tested for a phenotype during *Botrytis cinerea* infection as before. Lesion size was dramatically increased in 35S:*ANAC092* lines compared to Col 0, suggesting *ANAC092* contributes positively to *Botrytis cinerea* infection. This agrees with the phenotype observed for *anac092-1*, suggesting ANAC092 is a negative regulator of resistance to Botrytis.

Interestingly, *anac092-1* demonstrated reduced yellowing around the lesion site, while lines overexpressing *ANAC092* showed enhanced yellowing (figure 3.6). Yel-

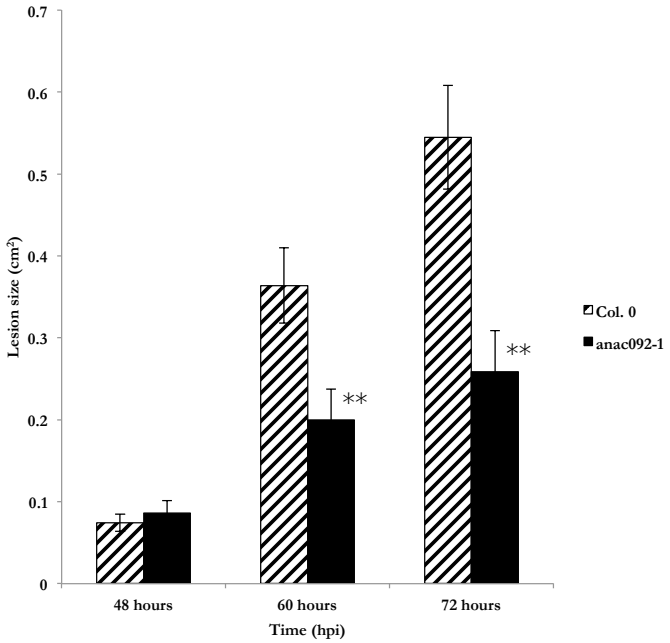


Figure 3.4: **Phenotype of *anac092-1* during *Botrytis cinerea* infection** Lesion size of Col 0 and *anac092-1* during *Botrytis cinerea* infection. Error bars represent SEM (N=30), ** indicates $p < 0.01$ in a Student's t-test.

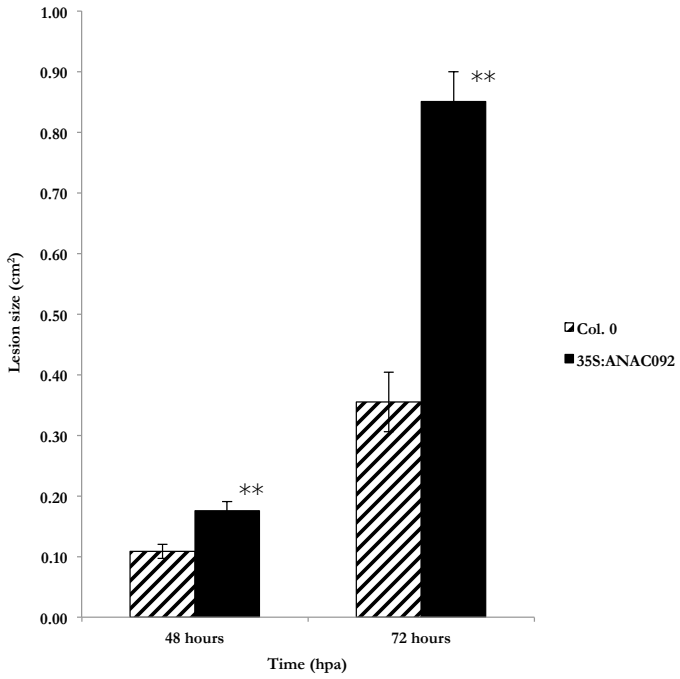


Figure 3.5: **Phenotype of 35S:*ANAC092* during *Botrytis cinerea* infection** Lesion size of Col 0 and 35S:*ANAC092* leaves infected with *Botrytis cinerea*. Error bars represent SEM (N=30), ** indicates $p < 0.01$ on a Student's t-test.

lowing is characteristic of a loss of chlorophyll, known as chlorosis, which is caused by the loss of green pigment during age-induced senescence. It is possible that in

wild type plants ANAC092 contributes to the spread of *Botrytis cinerea* infection by promoting the downregulation and degradation of photosynthetic components, in a manner similar to the decrease in photosynthesis that occurs during age-induced senescence. Infection by *Botrytis cinerea* has previously been shown to induce the expression of senescence-specific genes (Swartzberg *et al.*, 2008), therefore it is possible ANAC092 acts in this signalling pathway to induce senescence in response to *Botrytis cinerea* infection, which allows the lesion to spread further.

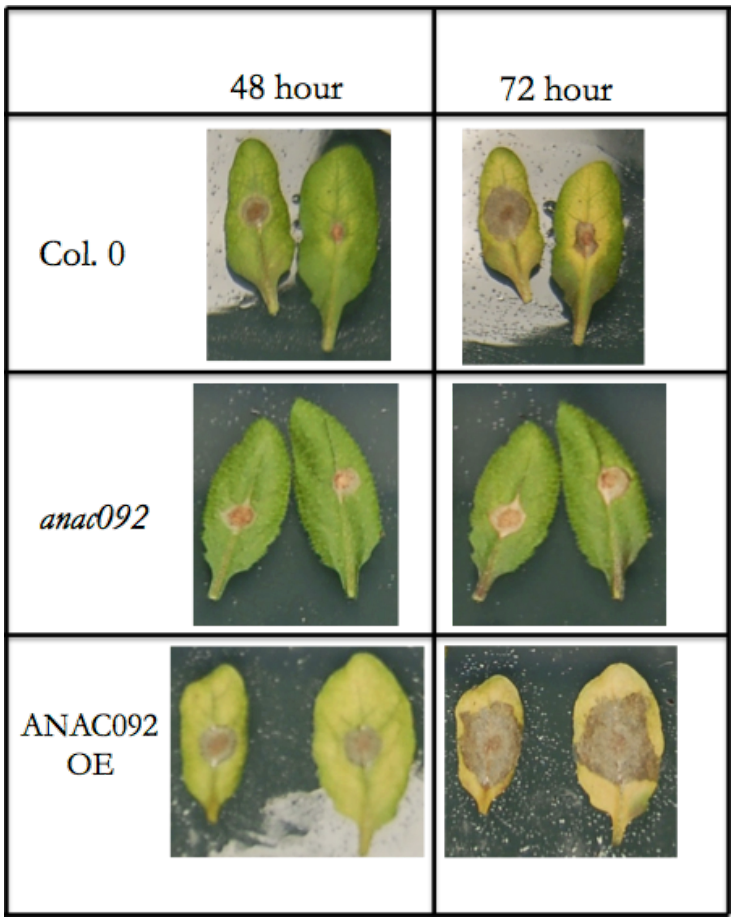


Figure 3.6: **Examples of detached Col 0, *anac092-1* and 35S:ANAC092 leaves infected with *Botrytis cinerea*** Examples of detached leaves of Col 0, *anac092-1* and 35S:ANAC092 infected with *Botrytis cinerea* at 48 and 72 hours post infection, showing changes in chlorosis around the lesion site in the transgenic lines.

3.2.1.4. *anac092-1* and overexpressors of *ANAC092* show altered susceptibility to *Hyaloperonospora arabidopsidis* (*Hpa*)

Botrytis cinerea is a necrotrophic pathogen of Arabidopsis, that is, it consumes the nutrients released from dead tissue (Tudzynski & Kokkelink, 2009). *Hyaloperonospora arabidopsidis* (*Hpa*) is an obligate hemibiotroph which draws its nutrients from a living leaf organ through infiltration of the extra and intracellular environ-

ment (Slusarenko & Schlaich, 2003; Coates & Beynon, 2010). Because of their differing lifestyles, responses to necrotrophs and biotrophs are correspondingly different (Glazebrook, 2005; Pieterse *et al.*, 2012). Like many other biotrophic organisms, *Hpa* secretes a number of effector proteins into the intracellular environment of an Arabidopsis cell and suppresses PTI. In response, Arabidopsis activates effector triggered immunity (ETI), the second layer of defence. Amongst other mechanisms, ETI suppresses JA/ET signalling through SA signalling pathways and attempts to induce the cell death through the hypersensitive response.

Since the mechanisms of response to biotrophs and necrotrophs are detrimental to each other, it was expected that transgenic plants of *ANAC092* would show contrasting phenotypes in response to Botrytis and *Hpa*. *anac092-1* plants had shown enhanced resistance to Botrytis, therefore it was expected they would show increased susceptibility to *Hpa*. Similarly, 35S:*ANAC092* plants were more susceptible to Botrytis than Col 0 and so it was expected they would be more resistant to *Hpa*.

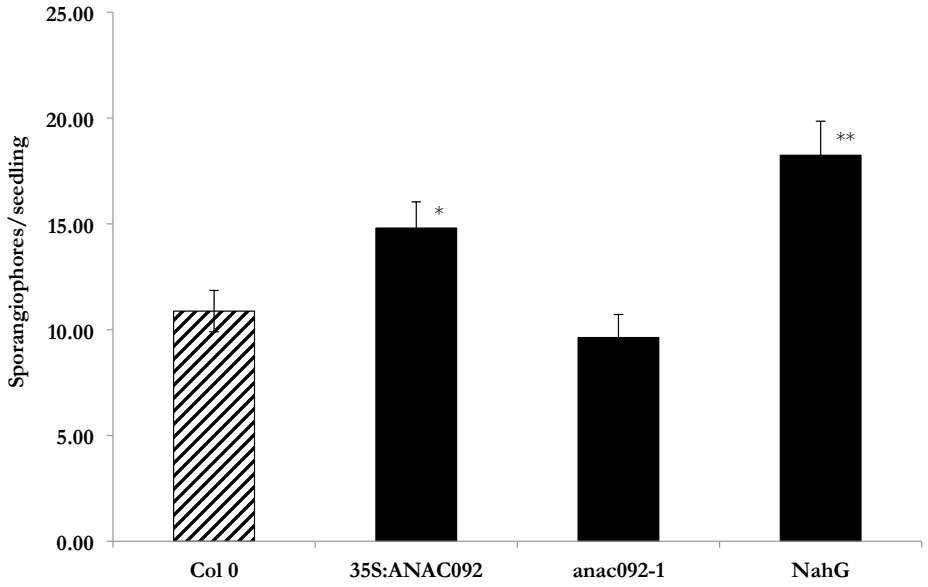


Figure 3.7: ***Hpa* susceptibility of Col 0, 35S:ANAC092, *anac092-1* and *NahG*** Sporangiophores emergent on Arabidopsis seedlings of Col 0, *anac092-1*, 35S:ANAC092 and *NahG* (Kachroo *et al.*, 2001). Error bars are standard error of the mean (N=40), * represents $p < 0.05$ and ** represents $p < 0.01$ from Student's t-test.

anac092-1 and 35S:*ANAC092* were tested for a phenotype during *Hpa* infection by Miss Lesley Foster. Seedlings of Arabidopsis were infected with *Hpa* and examined for emergence of sporangiophores as a measure of phenotype. Arabidopsis plants expressing *SALICYLATE HYDROXYLASE* (*NahG*), which degrades salicylic acid

and are therefore hypersusceptible to biotrophic infection were used as a positive control of infection (Kachroo *et al.*, 2001; Van Wees & Glazebrook, 2003).

Compared to the wild type plants, the *ANAC092* overexpressor showed a slight, but significant increase in sporangiophore numbers, suggesting it is slightly more susceptible to *Hpa* infection ($p = 0.01$). *anac092-1* had a reduced number of sporangiophores compared to wild type, but the decrease was not significant based on a Student's t-test ($p = 0.39$, figure 3.7). This suggests ANAC092 contributes to *Hpa* infection in a positive manner, meaning the phenotype demonstrated by *ANAC092* transgenic lines is similar during *Botrytis cinerea* infection and *Hyaloperonospora arabidopsidis* infection. This is incongruous with the necrotroph-biotroph paradigm normally demonstrated by resistance to plant pathogens, but may represent *ANAC092* is involved in more general stress response, as opposed to specific hormone based biotic stress response.

3.2.1.5. *ANAC092* expression can be driven from the upstream promoter region during *Botrytis cinerea* infection

It has been shown previously that expression of *GUS* can be driven from the 1500bp promoter region of *ANAC092* in senescent or salt-stressed tissue (Balazadeh *et al.*, 2010a), suggesting this promoter region is at least partly responsible for expression of *ANAC092.* The promoter region may also be responsible for expression of *ANAC092* during *Botrytis cinerea* infection, therefore the same plants (provided by Salma Balazadeh) were tested for expression of GUS after infection with Botrytis. 28 day old Arabidopsis expressing the 1500bp *ANAC092* promoter linked to GUS were infected with *Botrytis cinerea* as before, however multiple spots of Botrytis inoculum were used to provide more infection sites that could be used to determine GUS expression patterns. After infection, each leaf was taken and stained using X-Gluc for ß-glucuronidase expression at 24, 48 and 72 hours post infection.

Typical blue colouring was highly visible in the areas immediately adjacent to the infection site at 24, 48 and 72 hours post infection in two independent plant lines (figure 3.8). Some background staining was observed around the lesion site and on the mock treated samples. This may be because leaf senescence is being induced by detachment from the body of the plant, or the promoter of *ANAC092* has a certain background rate. These results suggest that the upstream genomic region of *ANAC092* is be capable of driving expression during *Botrytis cinerea* infection, in addition to developmental and salt-induced senescence.

3.2.1.6. *ANAC092* expression is diminished in *ein2-1* during *Botrytis cinerea* infection

ANAC092 expression is known to be induced in an EIN2 dependent manner during developmental senescence (Kim *et al.*, 2009). *ANAC092* expression does not

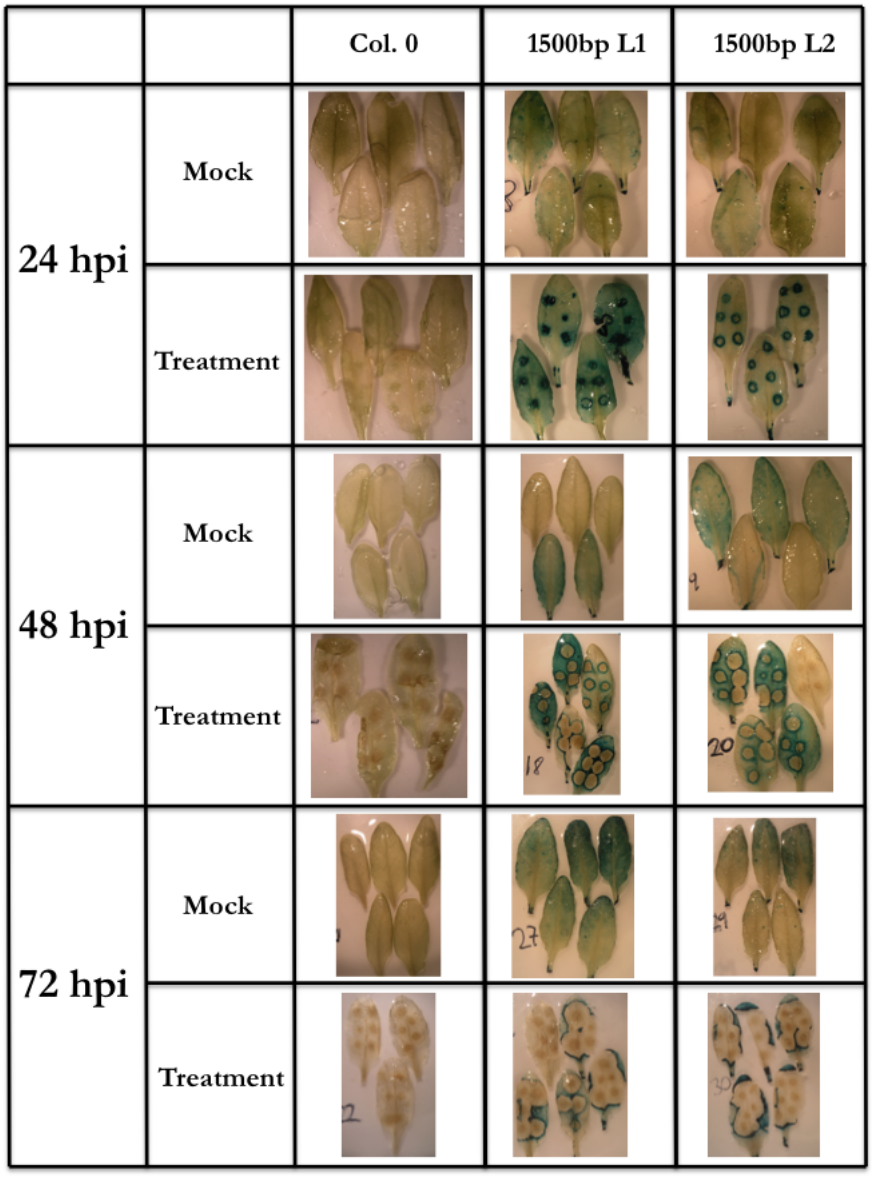


Figure 3.8: **GUS expression from the 1500bp *ANAC092* promoter during *Botrytis cinerea* infection** GUS staining of two independent plant lines (L1 and L2) expressing *ß-GLUCURONIDASE* from the 1500bp *ANAC092* promoter in a Col 0 background (Balazadeh *et al.*, 2010a) after infection with *Botrytis cinerea* at 24, 48 and 72 hours post infection (hpi). Blue staining indicates expression of *GUS* from the *ANAC092* promoter region, stained by X-Gluc. Dead tissue at the lesion site did not stain, therefore central regions are not blue.

accumulate in *EIN2* mutants during age-related senescence due to a trifurcate feed-forward mechanism involving *miR164* and EIN3 not being activated (Li *et al.*, 2013; Kim *et al.*, 2014). During *Botrytis cinerea* infection, EIN2 contributes to defence as shown by *ein2* mutants showing enhanced susceptibility (Thomma *et al.*, 1999; Ferrari *et al.*, 2003). This appears to be mediated by transcriptional changes induced by EIN2 post infection (AbuQamar *et al.*, 2006).

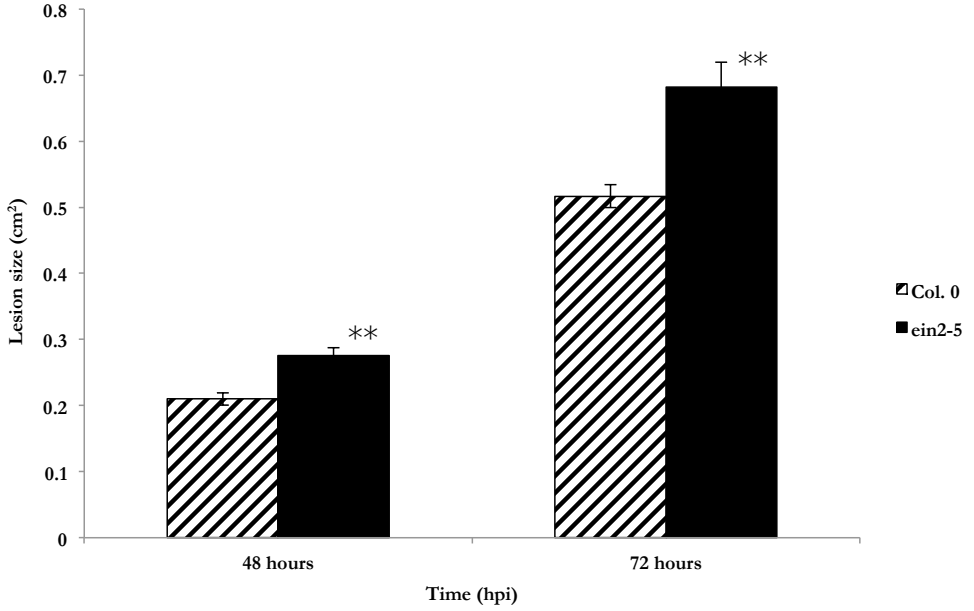


Figure 3.9: **Phenotype of *ein2-5* during *Botrytis cinerea* infection** Lesion size of *ein2-5* and Col 0 during *Botrytis cinerea* infection at 48 and 72 hours post infection. Error bars are standard error of the mean (N=30), ** indicates $p < 0.01$ on a Student's t-test.

ANAC092 expression was tested during *Botrytis cinerea* infection in an *EIN2* mutant, *ein2-5* (Alonso, 1999). Detached leaves from *ein2-5* and Col 0 were infected with multiple spots of *Botrytis cinerea* inoculum. *ein2-5* showed increased lesion size at *Botrytis* infection sites, in line with the susceptible phenotype of *ein2* reported in the literature (Thomma *et al.*, 1999; figure 3.9).

ANAC092 expression was tested in *ein2-5* and Col 0 at 24, 28 and 32 hours post infection using quantitative PCR. In Col 0, *ANAC092* accumulated in treated samples in accordance with the PRESTA timeseries data (figure 3.1). In mock samples, *ANAC092* expression was minimal and did not appear to increase. In *ein2-5*, *ANAC092* was not detected in mock treated samples, but was detected in treated samples, but at a significantly reduced rate compared to wild-type Col 0.

EIN2 is known to function in ethylene signalling (Alonso, 1999), *Botrytis cinerea* infection (Thomma *et al.*, 1999) and *ANAC092* signalling (Kim *et al.*, 2009; Li *et al.*, 2013; Kim *et al.*, 2014), therefore it is expected that *ANAC092* expression would be reduced in *EIN2* mutants during *Botrytis cinerea* infection. Indeed, *ANAC092*

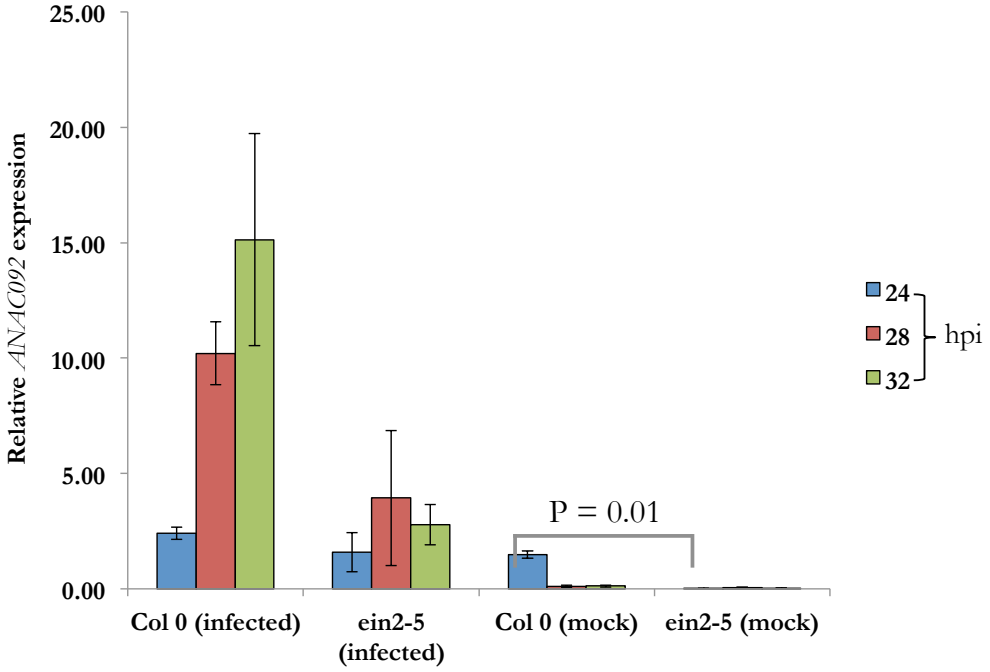


Figure 3.10: ***ANAC092* expression levels in *ein2-5* and Col 0 during *Botrytis cinerea* infection** qPCR of *ANAC092* relative to *PUX1* in Col 0 and *ein2-5* during both *Botrytis cinerea* infection and mock treatment. Error bars are standard error of the mean (N=3). p-values are shown where $p < 0.05$ with a Student's t-test. T-tests were only performed between lines or between treatments, not between treatments and lines.

expression was diminished in *EIN2* mutants in both mock and treated samples, but *ANAC092* did still accumulate in treated *ein2-5* samples (figure 3.10), suggesting an additional mechanism is involved in inducing *ANAC092* during *Botrytis cinerea* infection that does not involve EIN2. In mock treated samples, *ANAC092* was expressed at much lower levels, in both wild-type and *ein2-5*, suggesting *ANAC092* expression is at very low levels in unstressed 28 day old tissue.

3.2.2. Transcriptome changes in *anac092-1* during *Botrytis cinerea* infection

Although there is evidence to suggest that ANAC092 may promote localised senescence in the area surrounding the lesion site (figure 3.6), the transcriptional changes that occur in *anac092-1* compared to wild-type were determined to clarify the gene expression changes that may be induced by ANAC092 during *Botrytis cinerea* infection.

Detached *anac092-1* and Col 0 leaves were infected with *Botrytis cinerea* as before (figure 3.8) and gene expression changes were examined using qPCR and whole-genome microarrays.

3.2.2.1. *SAG12* is not expressed during *Botrytis cinerea* infection in *anac092-1*

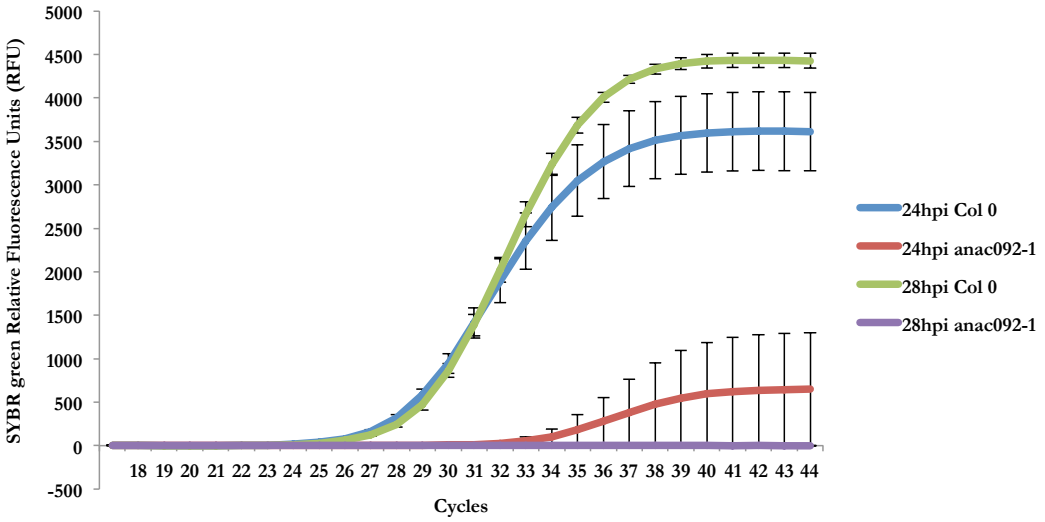
SAG12 is a one of the most well known senescence associated genes (SAGs, Lohman *et al.*, 1994; Grbic & Bleecker, 1995). *SAG12* encodes a cysteine protease that locates to senescence associated vacuoles which have high proteolytic activity, suggesting they may be involved in the degradation of proteins during senescence (Lohman *et al.*, 1994; Otegui *et al.*, 2005). Its expression is tightly linked to the loss of green pigment in tissue, suggesting SAVs and *SAG12* may be directly involved in degradation of chlorophyll and/or photosynthetic machinery.

Many senescence related genes accumulate over time as senescence progresses, however *SAG12* is not expressed at any level until late senescence (Lohman *et al.*, 1994; Noh & Amasino, 1999). This implies that *SAG12* is a senescence specific gene, in that it is expressed only in yellowing tissues. For comparison, the gene *SAG13* is expressed under a number of stress conditions that induce premature senescence such as dark or salt treatment (Weaver *et al.*, 1998). *SAG12* can be reduced in expression by treatment with senescence repressors such as cytokinin, however *SAG13* does not reduce during these treatments, suggesting *SAG13* expression is independent of the progress senescent process. *SAG12* is expressed in response to *Botrytis cinerea*, possibly induced by the pathogen as an infection mechanism (Swartzberg *et al.*, 2008). In addition, expression of *IPT* (which represses senescence) from the *SAG12* promoter confers resistance against *Botrytis cinerea* infection, suggesting senescence around the lesion site is critical to disease spread (Swartzberg *et al.*, 2008).

qPCR of *SAG12* during *Botrytis cinerea* infection at 24 and 28 hpi revealed low, but detectable expression of *SAG12* in Col 0 (figure 3.11A). Amplification of *SAG12* from *anac092-1* mRNA by PCR was not effective, suggesting the levels of *SAG12* transcript were insufficient (figure 3.11A). qPCR of *PUX1* used to normalise the data was relatively stable across all samples, suggesting qPCR was successful across all samples and did not bias the results against the *anac092-1* samples (figure 3.11B). This indicates SAG12 is not expressed in *anac092-1* during Botrytis infection, but is in Col 0.

The absence of *SAG12* expression indicates that *ANAC092* directly contributes to expression of *SAG12* during *Botrytis cinerea* infection. However, the mechanism is not immediately apparent. ANAC092 may bind to the promoter of *SAG12* and induce transcription of the gene, but the temporal expression patterns of both genes differ. During age-induced senescence, *ANAC092* transcript gradually increases over time, while *SAG12* shows a rapid increase in expression late in life-cycle of the leaf. Similarly, during a *Botrytis cinerea* infection *SAG12* is not expressed until late in the infection, while *ANAC092* is upregulated at approximately 22 hours post infection. If expression of *SAG12* was directly linked to *ANAC092*, it would be expected that an increase in *ANAC092* expression would be followed by an increase in *SAG12* expression, with a small lag time for synthesis of the transcript and protein. Instead,

(A) SAG12



(B) PUX1

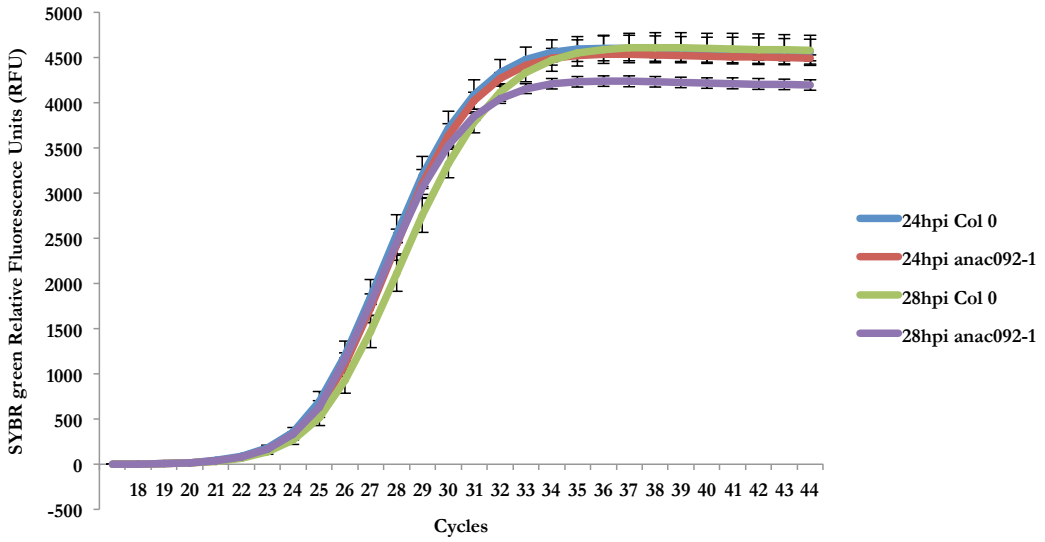


Figure 3.11: ***SAG12* expression during *Botrytis cinerea* infection in *anac092-1*** *SAG12* expression as determined by qPCR during *Botrytis cinerea* infection in Col 0 and *anac092-1* at 24 and 28 hours post infection. Shown are fluorescence curves generated during qPCR for (A) *SAG12* and (B) *PUX1* from all samples. (A) *SAG12* is transcript is amplified in a typical sigmoidal pattern during qPCR using Col 0 mRNA as a template, but is almost not amplified at all from mRNA extracted from *anac092-1*. (B) Amplification of *PUX1* using qPCR is stable from any template, indicating a successful reaction.

we see different profiles between the two genes. This might imply that *SAG12* is an indirect target of ANAC092. *ANAC092* regulates senescence, therefore *anac092-1* mutants exhibit delayed senescence. In turn, *SAG12* transcription is induced as part

of senescence, but senescence is diminished in *anac092-1*, therefore *SAG12* expression is reduced.

The repression of senescence in *anac092-1* implied by *SAG12* expression correlates with the reduced chlorosis around the lesion site exhibited by *anac092-1* plants during *Botrytis cinerea* infection (figure 3.6). It can be suggested that Botrytis induced senescence may be regulated through *ANAC092*, which is lost in *anac092-1* mutants.

3.2.2.2. Analysis of whole-genome expression level changes in *anac092-1* using microarray

ANAC092 may promote *SAG12* expression during *Botrytis cinerea* infection, but it may also regulate a number of other genes. To determine transcriptome changes in *anac092-1* compared to Col 0 that may be responsible for the phenotype, gene expression levels were determined using a whole-genome microarray.

The RNA samples previously used for qPCR were analysed for gene expression changes using NimbleGen 12x135k microarrays (Roche NimbleGen). Due to cost constraints, RNA from three biological replicates were combined at equal quantities and analysed on a single microarray.

3.2.2.3. Identification of genes differentially expressed in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection

Gene expression changes can often be analysed by examining the log fold change of fluorescence at each microarray probe relating to a gene. This gives a relative ratio of mRNA present in the *anac092-1* material compared to the Col 0 material, which can indicate large differences in gene expression. Many genes were expressed at different levels in *anac092-1* compared to Col 0. These gene expression changes may be responsible for the strong phenotype observed during Botrytis infection. Table 3.1 and 3.2 show genes expressed at eight-fold up or down in *anac092-1* compared to Col 0 at both 24 and 28 hours during *Botrytis cinerea* infection. *SAG12* appears to be downregulated by a particularly large amount, which correlates with the qPCR data observed above (figure 3.11). Similarly, *PROTEIN ACID PHOSPHATASE 20* (*PAP20*) and *NICOTIANAMINE SYNTHASE 3* (*NAS3*) are expressed at dramatically reduced levels in *anac092-1* compared to Col 0. *PAP20* is a predicted serine/threonine protease, while NAS3 is involved in synthesis of nicotianamine, a metal chelator for redistribution of iron (Pianelli *et al.*, 2005). Neither have been directly implicated in response to Botrytis or senescence, however in the PRESTA timeseries data expression of *PAP20* increases during senescence and *Botrytis cinerea* infection, while expression of *NAS3* is stable during both (figure 3.12). Perhaps, senescence and Botrytis induced expression of *PAP20* is dependent on the action of ANAC092.

ATG	Gene Name	Log_2 fold change at 24hpi	Log_2 fold change at 28hpi
AT5G45890	SAG12	-6.34	-5.20
AT3G28899		-5.13	-6.04
AT3G52780	PAP20	-5.50	-5.16
AT1G09240	NAS3	-6.42	-3.17
AT5G64190		-5.26	-4.10
AT2G19500	CKX2	-5.01	-3.61
AT2G23970		-4.70	-3.67
AT2G20030		-3.99	-3.92
AT5G50610		-4.37	-3.28
AT4G32810	CCD8	-4.31	-3.24
AT3G22570		-3.45	-4.10

Table 3.1: **Genes expressed 8-fold down in *anac092-1* during *Botrytis cinerea* infection at 24 and 28 hours post infection** Genes expressed at log_2 fold change $<$ -3 during *Botrytis cinerea* infection at both time points, as determined by whole genome microarray.

ATG	Gene Name	Log_2 fold change at 24 hpi	Log_2 fold change at 28 hpi
AT5G46295		5.83	4.03

Table 3.2: **Genes expressed 8-fold up in *anac092-1* during *Botrytis cinerea* infection at 24 and 28 hours post infection** Genes expressed at log_2fold change $>$ 3 during *Botrytis cinerea* infection at both time points, as determined by whole genome microarray.

3.2.2.4. Identification of genes differentially expressed by 2-fold change

Using fold change is a more crude method of identification of differentially expressed genes then statistical tests, but it may be more appropriate with limited biological replicates. Genes were determined to be differentially expressed at log (base 2) fold change $> \pm 1$. This identified a number of genes as differentially expressed in different combinations (table 3.3). Between 1080 and 2065 genes were determined to be differentially expressed at any time point, either up or down. More genes were decreased in expression than increased at both time points, which correlates with the function of ANAC092 to be a transcriptional activator (Olsen *et al.*, 2005a; Matallana-Ramirez *et al.*, 2013). There was limited overlap between the time points, suggesting the gene expression levels are variable and not consistent. As such, genes that were expressed at 2-fold down at both time points were taken as a positive result.

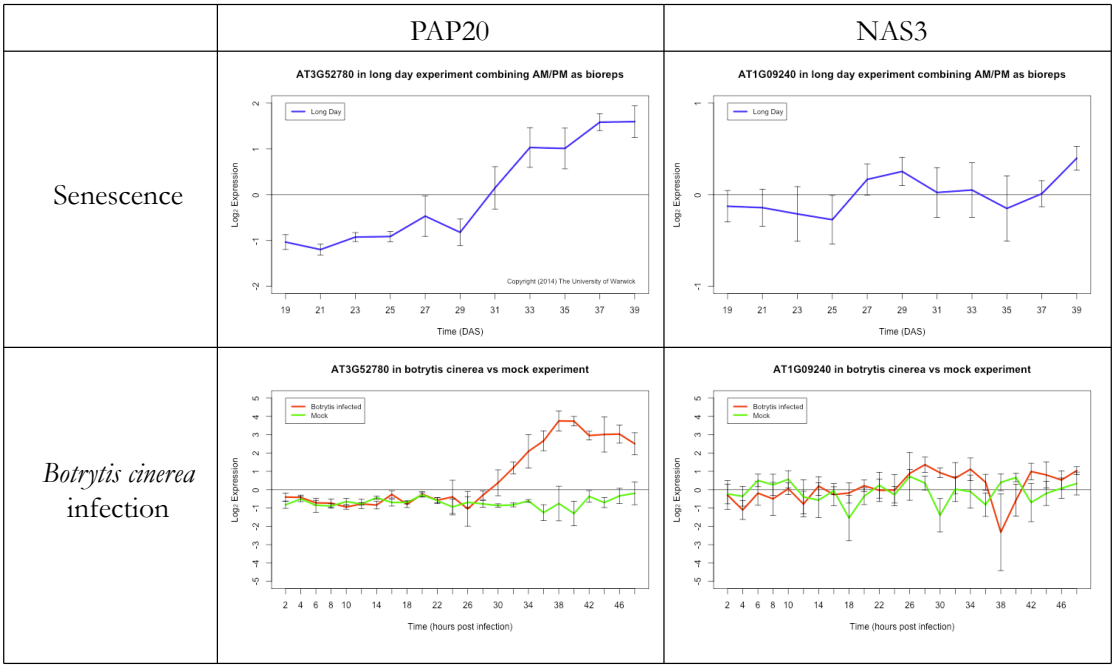


Figure 3.12: **Expression of *PAP20* and *NAS3* during age-induced senescence and *Botrytis cinerea* infection** Expression of *PAP20* and *NAS3* as determined by the PRESTA time course microarrays during senescence and *Botrytis cinerea* infection. Expression of PAP20 increases in both conditions while expression of *NAS3* is stable during both.

	24hpi	28hpi	Both
Up	1761	1080	191
Down	2065	1502	349

Table 3.3: **Genes expressed at 2 fold change in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection** Table of genes differentially expressed in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection at 24 hpi, 28 hpi and both time points.

3.2.2.5. GO terms related to abscission, cell wall disassembly and starvation are associated with genes differentially downregulated by 2-fold in *anac092-1* compared to Col 0

Analysis of GO terms overrepresented in sets of differentially expressed genes can identify shared functions of those genes. Overrepresentation of GO terms associated with the genes expressed at 2-fold change in *anac092-1* compared to Col 0 was performed using the Biological Networks Gene Ontology tool (BiNGO; Maere *et al.*, 2005).

No GO terms were associated with genes that were upregulated in *anac092-1*, but a number of GO terms were associated with downregulated genes including abscission and cell wall disassembly (table 3.4). Functionally redundant GO terms were removed to ensure there that similar GO terms were not present due to the same

sets of genes. Cell wall associated GO terms were also identified as overrepresented in genes upregulated in an *ANAC092* inducible-overexpressor in Balazadeh *et al.* (2010a). These same GO terms were observed to be overrepresented in the genes differentially expressed in *anac092-1* (table 3.4). This may indicate ANAC092 promotes cell wall disassembly, which is diminished in the *anac092-1* mutant. How this contributes to the Botrytis resistant phenotype of *anac092-1* is unclear.

GO-ID	p-value	Corr. p-value	Description
9838	1.91E-06	1.98E-03	Abscission
15698	2.93E-05	4.35E-03	Inorganic Anion Transport
44277	1.83E-05	4.35E-03	Cell Wall Disassembly
41	6.09E-05	7.92E-03	Transition Metal Ion Transport
9267	1.16E-04	1.35E-02	Cellular Response to Starvation
15706	0.000533	0.0326	Nitrate Transport
10227	0.000659	0.0381	Floral Organ Abscission

Table 3.4: **GO terms associated with genes downregulated in *anac092-1* during *Botrytis cinerea* infection** GO terms overrepresented in the 349 genes downregulated by 2 fold in *anac092-1* compared to Col 0 at 24 and 28 hours post infection by *Botrytis cinerea*, as determined by BiNGO (Maere *et al.*, 2005).

3.2.2.6. A number of premature-senescence related GO terms are associated with genes expressed at 2-fold up or down in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection

No GO terms were identified to be overrepresented in the 191 genes increased in transcription 2 fold in *anac092-1* compared to Col 0. This may be expected considering ANAC092 has been identified to act as a transcriptional activator (Matallana-Ramirez *et al.*, 2013), therefore elimination of a functioning transcript is more likely to reduce global gene expression than increase it. However, ANAC092 has also been shown to act as a repressor of transcription by inhibiting the action of other transcription factors (Rauf *et al.*, 2013). Therefore it is possible ANAC092 functions through activation and repression of target genes, in which case the phenotype observed in *anac092-1* may be a result genes expressed up and down in *anac092-1.*

All genes expressed at 2-fold change up or down in *anac092-1* compared to Col 0 were analysed for overrepresentation of GO terms via BiNGO (Maere *et al.*, 2005). A number of GO terms were identified relating to a number of different biological processes (table 3.5). These GO terms were similar to the terms associated with the genes downregulated in *anac092-1*, but appeared to have lower p-values due to the increased numbers of genes in each GO term set. This indicates the genes upregulated in *anac092-1* are of a similar function to the genes downregulated in

anac092-1.

GO-ID	p-Value	Corr. p-Value	Description
9838	1.99E-07	2.98E-04	Abscission
44277	3.16E-06	1.00E-03	Cell Wall Disassembly
9267	1.89E-04	2.49E-02	Cellular Response to Starvation
30001	2.46E-04	2.62E-02	Metal Ion Transport
5576	2.25E-04	2.62E-02	Extracellular Region
3993	6.20E-04	4.58E-02	Acid Phosphatase Activity
15706	7.07E-04	4.67E-02	Nitrate Transport

Table 3.5: **GO terms associated with genes differentially expressed in *anac092-1* during *Botrytis cinerea* infection** GO terms overrepresented in both genes that were upregulated and downregulated by 2-fold in *anac092-1* compared to Col 0 at 24 and 28 hours post infection by *Botrytis cinerea.*

The GO terms represented a spectrum of biological processes, however most had some relevance to senescence. Abscission is a key process involving the separation of plant cells from each other, primarily for loss of leaves and flowers (Estornell *et al.*, 2013). The role abscission plays in response to *Botrytis cinerea* has not been studied in great detail, but it is possible abscission is induced to remove infected organs, which may be mediated by *ANAC092.*

Cell wall disassembly is a process involved in senescence as part of the degradation of leaves, with cell wall degrading enzymes increasing in expression during senescence (Park *et al.*, 1998; Yoshida *et al.*, 2001; Breeze *et al.*, 2011). Similarly, the cell wall is degraded by Botrytis as an infection mechanism, to allow the fungus to infiltrate the leaf tissue (Have *et al.*, 1998; Wubben *et al.*, 1999; Kars *et al.*, 2005; Espino *et al.*, 2010). Perhaps *Botrytis cinerea* induces *ANAC092* expression to promote senescence related cell wall degradation to damage leaf tissue, which facilitates the spread of the pathogen.

Response to starvation is a GO term with relevance to senescence. Carbon starvation of Arabidopsis cell cultures induces causes programmed cell death (Swidzinski *et al.*, 2002), while dark-treatment halts photosynthesis and therefore leads to a deficit of carbon molecules and induces senescence (Buchanan-Wollaston *et al.*, 2005). Therefore response to nutrient starvation can be considered a component of premature senescence. In *anac092-1*, transcription of genes associated with these processes is altered in expression, therefore maybe *ANAC092* is involved in promotion of genes associated with premature senescence during infection with *Botrytis cinerea* infection.

3.2.2.7. Motifs associated with gene expression changes in *anac092-1*

Genes which are downregulated in *anac092-1* may be induced in expression by ANAC092 acting directly on their promoter regions in wild type Col 0. As such, they may share a sequence in their upstream promoter regions that the ANAC092 protein recognises and binds to. ANAC092 has also been shown to repress GOLDEN2-LIKE (GLK1 and 2) transcription factors action through protein binding, therefore ANAC092 may suppress the action of other transcription factors which recognise alternative sequences. In either instance, it is expected genes that are consistently downregulated or upregulated in *anac092-1* may share sequence conservation in their promoter regions.

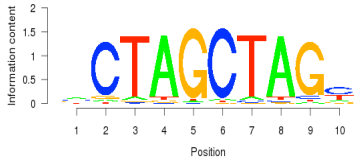
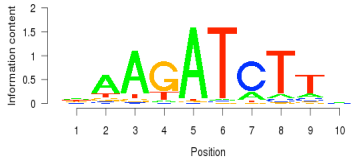
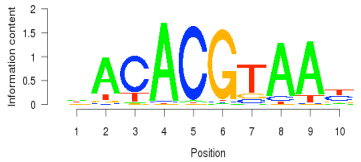
Feature Name	Motif Logo	Number of instances	Significance
STY1		75	0.00357
ARR11		75	0.00357
ANAC055		74	0.00571

Table 3.6: **Transcription Factor recognition sequences associated with promoter regions of genes upregulated in *anac092-1* during *Botrytis cinerea* infection** The promoter regions of 191 genes upregulated by 2-fold in *anac092-1* during *Botrytis cinerea* infection at both 24 and 28 hours post infection. Promoter region is defined as intergenic region extending from transcription start site to nearest coding sequence, as defined in TAIR10. Overrepresentation of motifs detected using Motif Enrichment Tool (Blatti & Sinha, 2014). Original transcription factor binding motifs identified with protein binding microarrays in Franco-Zorrilla *et al.* (2014). Table indicates transcription factor and associated motif that is overrepresented, alongside number of times the motif appears in the promoter regions of the 191 genes and significance as determined by MET.

The Motif Enrichment Tool (MET) takes collections of characterised transcription factor motifs and scans for their occurrence in the promoter regions of gene sets provided by the user (Blatti & Sinha, 2014). For Arabidopsis, MET uses motifs identified through a series of protein binding microarray experiments, which included ANAC055 and ANAC019 (Franco-Zorrilla *et al.*, 2014). In this experiment, the

recognition sequence of ANAC055 was determined to be TTNCGTGT. ANAC092 has been seen to recognise a CGT[G/A] sequence in selex (Olsen *et al.*, 2005a), EMSA (Jensen *et al.*, 2010) and ChIP (Matallana-Ramirez *et al.*, 2013), but also a TTNCGT[G/A] sequence in protein binding microarrays (Lindemose *et al.*, 2014). Since the ANAC092 recognition sequences are similar to the ANAC055 recognition sequence, it may be expected that a NAC recognition sequence is overrepresented in the promoter regions of genes differentially expressed in *anac092-1.*

The promoter regions of genes expressed at 2-fold change at both time points were analysed for conserved motifs in MET. Promoter regions were analysed as upstream from the transcription start site of the gene until the next coding sequence. Arabidopsis TAIR10 mappings were used for genome references and as a background.

There were no significantly overrepresented transcription factor binding motifs associated with the promoter regions of genes downregulated in *anac092-1.* However, 118 of the 349 sequences did contain an ANAC055 recognition sequence (TTACGT), suggesting some of these genes were partially regulated by ANAC092.

For the genes upregulated in *anac092-1* during *Botrytis cinerea* infection, a number of motifs were associated with their promoter region (table 3.6). In particular, the binding motif for STY1, ARR11 and ANAC055 were seen as overrepresented. The enhanced presence of these motifs on promoter regions may suggest that ANAC092 is involved in regulation through them. However, since the genes are upregulated in the *ANAC092* mutant, it suggests ANAC092 represses transcription through these motifs in wild type Col 0. ANAC092 has been seen to bind to GLK1/2 transcription factors and repress their action (Rauf *et al.*, 2013) and it is known NAC proteins can heterodimerise (Olsen *et al.*, 2005a). Perhaps, ANAC092 binds to and represses transcription factors such as STY1, ARR11 and ANAC055. In the *anac092-1* mutant, its absence allows targets of those transcription factors to increase.

3.2.2.8. Comparison to the known ANAC092 regulatory network

In Matallana-Ramirez *et al.* (2013), Arabidopsis mesophyll protoplasts were transformed with a construct constitutively expressing *ANAC092* and changes in transcriptome were determined using an Affymetrix microarray. This work identified 831 genes as differentially expressed by a 2-fold cutoff when the overexpressors were compared to empty vector treated protoplasts. The genes identified as differentially expressed in this experiment were compared with the expression of genes in *anac092-1* during *Botrytis cinerea* infection (figure 3.13). 14 genes were not present as probes on the NimbleGen microarray and were therefore excluded from analysis.

A number of genes were identified as differentially expressed in both Matallana-Ramirez *et al.* (2013) and this experiment (table 3.7). Of the 45 genes identified as differentially expressed in both Matallana-Ramirez *et al.* (2013) and in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection, 40 were expressed at $\log_2$ fold

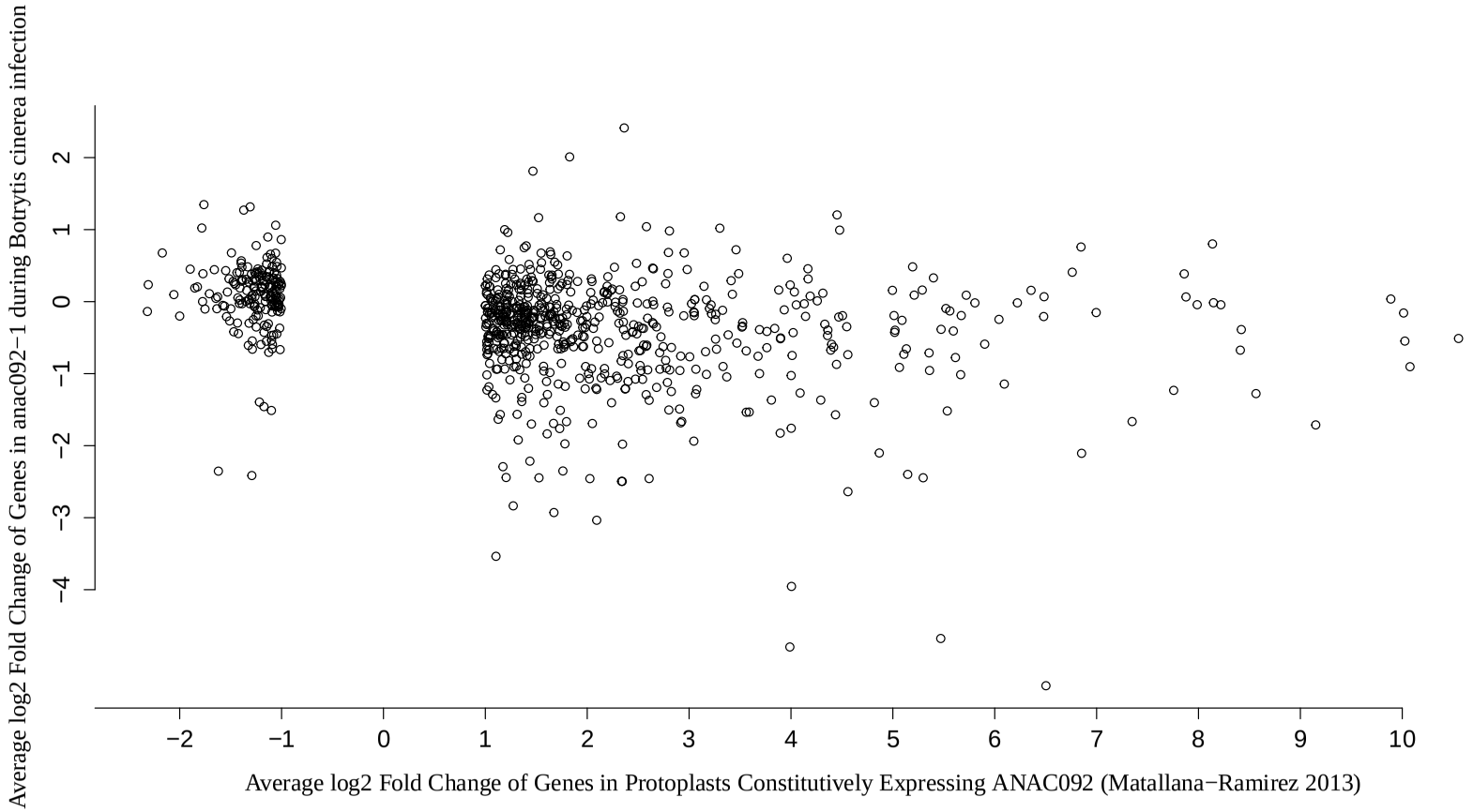


Figure 3.13: **Expression of genes in *anac092-1* during *Botrytis cinerea* infection compared to expression in protoplasts expressing *ANAC092*** Log_2 fold-change of gene expression in protoplasts overexpressing ANAC092 as determined by Matallana-Ramirez *et al.* (2013) compared to log_2 fold-change of genes in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection. Expression of genes determined in Matallana-Ramirez *et al.* (2013) is shown along the x-axis, while average expression of genes determined in *anac092-1* during *Botrytis cinerea* infection is shown on the y-axis. An average of 24 and 28 hours post infection is used.

change < -1 in *anac092-1* during *Botrytis cinerea* infection at both time points, suggesting that ANAC092 positively contributes their expression level in protoplasts and during *Botrytis cinerea* infection. One gene was differentially expressed down in Matallana-Ramirez *et al.* (2013) and expressed up in *anac092-1* compared to Col 0 during *Botrytis cinerea* infection (see table 3.7), suggesting ANAC092 negatively regulates the expression of this gene.

Table 3.7: **Genes differentially expressed in protoplasts expressing *ANAC092* and *anac092-1* during Botrytis infection** Genes identified as differentially expressed in protoplasts constitutively expressing *ANAC092* (compared to an empty vector) in Matallana-Ramirez *et al.* (2013) and in *anac092-1* during *Botrytis cinerea* infection compared to Col 0, as determined by microarray. In both cases, 2-fold change is used as a significance cut-off. All expression values are expressed as $\log_2$fold change from the control.

AGI Identifier	Annotation	Protoplasts expressing *ANAC092*	*anac092-1* (24hpi)	*anac092-1* (28hpi)
AT3G52780	ATPAP20/PAP20	6.503	-5.50	-5.16
AT1G09240	nicotianamine synthase, putative	3.989	-6.42	-3.17
AT5G64190	similar to unknown protein AT2G40390.1	5.470	-5.26	-4.10
AT2G20030	zinc finger family protein	4.005	-3.99	-3.92
AT4G25000	AMY1 (ALPHA-AMYLASE-LIKE); alpha-amylase	1.105	-5.23	-1.84
AT4G30670	unknown protein	2.093	-2.82	-3.25
AT2G03850	LEA domain-containing protein	1.673	-3.54	-2.32
AT5G42500	disease resistance-responsive family protein	4.559	-3.63	-1.65
AT3G15830	phosphatidic acid phosphatase-related / PAP2-related]	2.344	-3.91	-1.08
AT1G14780	similar to unknown protein AT4G24290.2	2.337	-2.58	-2.41
AT2G32660	LRR family protein	2.026	-3.74	-1.17
AT5G02420	similar to unknown protein AT1G08180.1	2.608	-3.78	-1.14
AT2G01890	PAP8	1.527	-3.16	-1.73
AT2G41850	endo-polygalacturonase, putative	5.298	-2.60	-2.29
AT2G42900	similar to Os05g0582000	5.146	-3.56	-1.23
AT2G39710	aspartyl protease family protein	1.173	-2.81	-1.77
AT2G40670	ARR16	4.867	-2.40	-1.81
AT1G68765	IDA	1.781	-1.90	-2.05

AT5G39520	similar to unknown protein AT5G39530.1	3.047	-2.61	-1.26
AT3G12700	aspartyl protease family protein	1.323	-1.59	-2.25
AT1G05450	lipid transfer protein (LTP)-related	3.894	-1.69	-1.97
AT5G26940	exonuclease family protein	4.003	-1.94	-1.58
AT1G07590	pentatricopeptide (PPR) repeat-containing protein	2.051	-2.31	-1.07
AT4G18340	glycosyl hydrolase family 17 protein	1.669	-1.71	-1.67
AT4G01430	nodulin MtN21 family protein	2.918	-1.75	-1.61
AT1G73750	similar to unknown protein AT1G15060.1	2.928	-1.90	-1.42
AT1G48260	CIPK17 (CIPK17); kinase	4.437	-1.76	-1.38
AT1G51830	ATP binding kinase	1.313	-1.15	-1.98
AT5G04390	zinc finger (C2H2 type) family protein	3.590	-1.91	-1.15
AT1G62760	invertase/pectin methylesterase inhibitor	5.534	-1.61	-1.42
AT2G05910	similar to unknown protein AT5G20640.1	2.801	-1.46	-1.55
AT5G54130	calcium ion binding	2.239	-1.29	-1.52
AT5G54130	calcium ion binding	1.575	-1.29	-1.52
AT2G44010	similar to unknown protein AT3G59880.1	1.358	-1.33	-1.45
AT3G45010	SCPL48	4.292	-1.41	-1.32
AT1G15380	lactoylglutathione lyase family protein	2.579	-1.27	-1.31
AT5G47740	similar to protein kinase family protein AT2G45910.1	1.609	-1.11	-1.47
AT1G23560	similar to unknown protein AT1G70480.2	3.064	-1.29	-1.27
AT5G24860	FPF1 (FLOWERING PROMOTING FACTOR 1)	4.090	-1.43	-1.11
AT3G24460	TMS membrane family protein	2.086	-1.16	-1.25
AT2G04040	ATDTX1	-1.762	1.63	1.06

Considering there are two independent sources of data, using differing techniques it seems likely expression of these genes is directly linked to *ANAC092* expression. The majority of the genes identified have not been studied directly, but many are predicted to act as kinases, phosphatases, proteases and transcription factors. Kinases and phosphatases are critical regulators of protein function, through addition or removal of phosphate groups. Similarly, transcription factors have a regulatory

role for the transcription of other genes. This may indicate one critical function of ANAC092 is regulating other regulators, thus initiating a downstream signalling cascade. However, without explicit understanding of each of the target genes of *ANAC092* it is difficult to clarify the exact role of ANAC092. Studying the molecular function of these genes is critical for to determine the direct role ANAC092 has on *Botrytis cinerea* stress response.

Finally, 17 genes were identified to be differentially expressed in protoplasts overexpressing *ANAC092* and an inducible overexpressor of *ANAC092* two and five hours post induction (Matallana-Ramirez *et al.*, 2013). The consistent change in transcription levels of these genes in experiments with modified *ANAC092* expression levels lead the authors to suggest these were direct targets of ANAC092. The expression of these 17 genes in *anac092-1* during *Botrytis cinerea* infection was examined.

ATG	Name	24 hpi	28 hpi
AT5G39520	Protein of unknown function (DUF1997)	-2.61	-1.26
AT5G13170	SAG29	-4.93	1.50
AT1G73750	alpha/beta hydrolase	-1.90	-1.42
AT1G48260	CIPK17	-1.76	-1.38
AT1G02470	Polyketide cyclase/dehydrase and lipid transport protein	-2.08	-0.94
AT3G45010	scpl48	-1.41	-1.32
AT3G13672	TRAF-like protein	-2.74	0.69
AT1G02660	alpha/beta-Hydrolases y protein	-0.75	-0.77
AT4G19810	ChiC - Glycosyl hydrolase protein	-0.54	-0.88
AT1G74010	Ca-dependent phosphotriesterase protein	-0.92	-0.31
AT1G11190	BFN1	-1.20	-0.81
AT1G26820	RNS3	-1.40	0.38
AT4G18425	Protein of unknown function (DUF679)	-1.66	1.35
AT4G04490	CRK36 - cysteine-rich RECEPTOR-like protein kinase 36	-0.24	0.47
AT2G31945	Unknown protein	-0.17	0.74
AT2G47950	Unknown protein	1.08	0.19
AT1G80450	VQ motif-containing protein	0.97	0.34

Table 3.8: **Expression of ANAC092 putative targets during *Botrytis cinerea* infection** Expression of genes identified as potential targets of ANAC092 in Matallana-Ramirez *et al.* (2013) during *Botrytis cinerea* infection at 24 and 28 hours post infection in *anac092-1*, displayed as $\log_2$ fold change. Green illustrates genes the gene is expressed fold change $<$ -2 in *anac092-1*, while red indicates the gene is expressed fold change $>$ 2 in *anac092-1*. Yellow shading indicates the gene was identified as differentially expressed during age-induced senescence in Breeze *et al.* (2011).

11 of the 17 genes identified as differentially expressed in Matallana-Ramirez *et al.* (2013) were identified as differentially expressed in *anac092-1* during *Botrytis cinerea* infection, as determined by $\log_2$ fold change $\geq \pm 1$. If the transcription of these genes is promoted by ANAC092, then it would be expected their expression would diminish in plants lacking a functional *ANAC092* gene. 10 genes were significantly

downregulated at 24 hpi, however only 4 genes were significantly downregulated at 28 hpi. This suggests that many ANAC092 regulated genes are downregulated early in the Botrytis infection, but recover quickly over the next 4 hours. This possibly indicates a redundant mechanism of expression for these genes, which allows them to be expressed despite the absence of ANAC092.

This affect is exemplified by *SAG29/SWEET15*, which is reduced in expression 30 fold in *anac092-1* at 24 hpi, but expressed 3 fold higher in *anac092-1* at 28 hpi (table 3.8). SAG29 is a plasma membrane localised MtN3/SALIVA protein (Seo *et al.*, 2011), whose expression increases during age-induced senescence (Gepstein *et al.*, 2003). In young plants it is primarily expressed in lateral roots, while in mature plants it is primarily expressed in flowers and siliques. In leaves, it is only expressed in senescent areas of the leaf (Seo *et al.*, 2011). *SAG29* is also expressed following salt-stress and ABA treatment (Seo *et al.*, 2011). In the PRESTA timeseries data, *SAG29* increases during senescence and increases in Botrytis infected tissue compared to mock treated samples (figure 3.14). These expression patterns correlate with the expression patterns exhibited by *ANAC092* (Kim *et al.*, 2009; Balazadeh *et al.*, 2010a), while expression of *SAG29* is affected by manipulation of *ANAC092* expression level (Matallana-Ramirez *et al.*, 2013), suggesting *SAG29* is a direct target of ANAC092.

Furthermore, plants constitutively expressing *SAG29* show accelerated senescence and are hypersensitive to salt-stress (Seo *et al.*, 2011), similar to *ANAC092* overexpressing plants (Balazadeh *et al.*, 2010a). However, expression of *SAG29* appears to be higher in *anac092-1* at 28hpi during *Botrytis cinerea* infection. This would imply ANAC092 may be repressing *SAG29* expression during Botrytis infection, allowing the expression to be higher in Col 0. This affect may be limited by the limited number of samples, further study would be needed to confirm *SAG29* is a direct target of ANAC092.

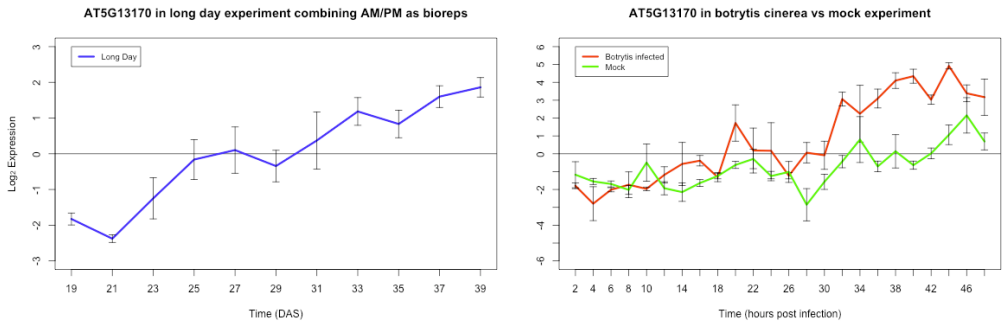


Figure 3.14: **Expression of SAG29 during *Botrytis cinerea* infection and age-induced senescence** Expression of *SAG29* in the PRESTA timeseries data during age-induced senescence and *Botrytis cinerea* infection.

3.2.2.9. Known senescence and Botrytis related stress responsive gene

In order to identify whether ANAC092 promotes senescence during *Botrytis cinerea* infection, the expression levels of a number of stress responsive genes were identified from literature and analysed for changes in *anac092-1* during Botrytis infection (figure 3.15). *BFN1* was included as a known target of ANAC092 (Matallana-Ramirez *et al.*, 2013). Most genes are only mildly affected by perturbation of *ANAC092* transcript level, however a small group of genes are downregulated at both time points including *SAG12*, *LHCA6*, *BFN1* and *ATSGR2*. This suggests that ANAC092 regulates a subset of senescence related genes during *Botrytis cinerea* infection. *MPK6*, *BIK1* and *WRKY6* were included as known Botrytis response genes, however these did not appear to be differentially expressed in *anac092-1* compared to Col 0 at either time point.

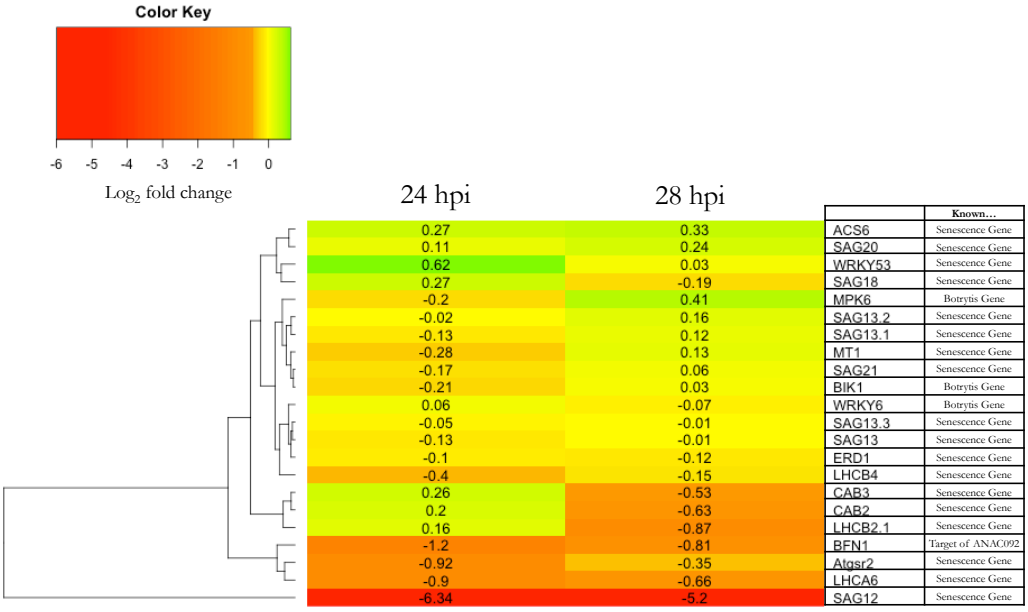


Figure 3.15: **Expression of known senescence and *Botrytis* related genes in *anac092-1* compared to Col 0** Log_2fold change of genes known to be involved in senescence or response to *Botrytis cinerea* in *anac092-1* compared to Col 0. Genes have been clustered using hierarchical clustering with their expression levels. Known role of the gene is included.

CAB2 (*CHLOROPHYLL A/B BINDING PROTEIN*), *CAB3* and *LHCB2* (*LIGHT HARVESTING BINDING COMPLEX 2*) are expressed at higher levels in *anac092-1* at 24 hpi, then lower at 28 hpi (figure 3.15). CAB and LHCB proteins form part of the photosynthetic apparatus in Arabidopsis (Hensel *et al.*, 1993). During senescence, photosynthesis declines and concurrently *CAB* and *LHCB* gene expression levels decrease (Lohman *et al.*, 1994).

In an attempt to investigate this further, expression of genes related to photosynthetic machinery were examined. Gene models associated with the GO term

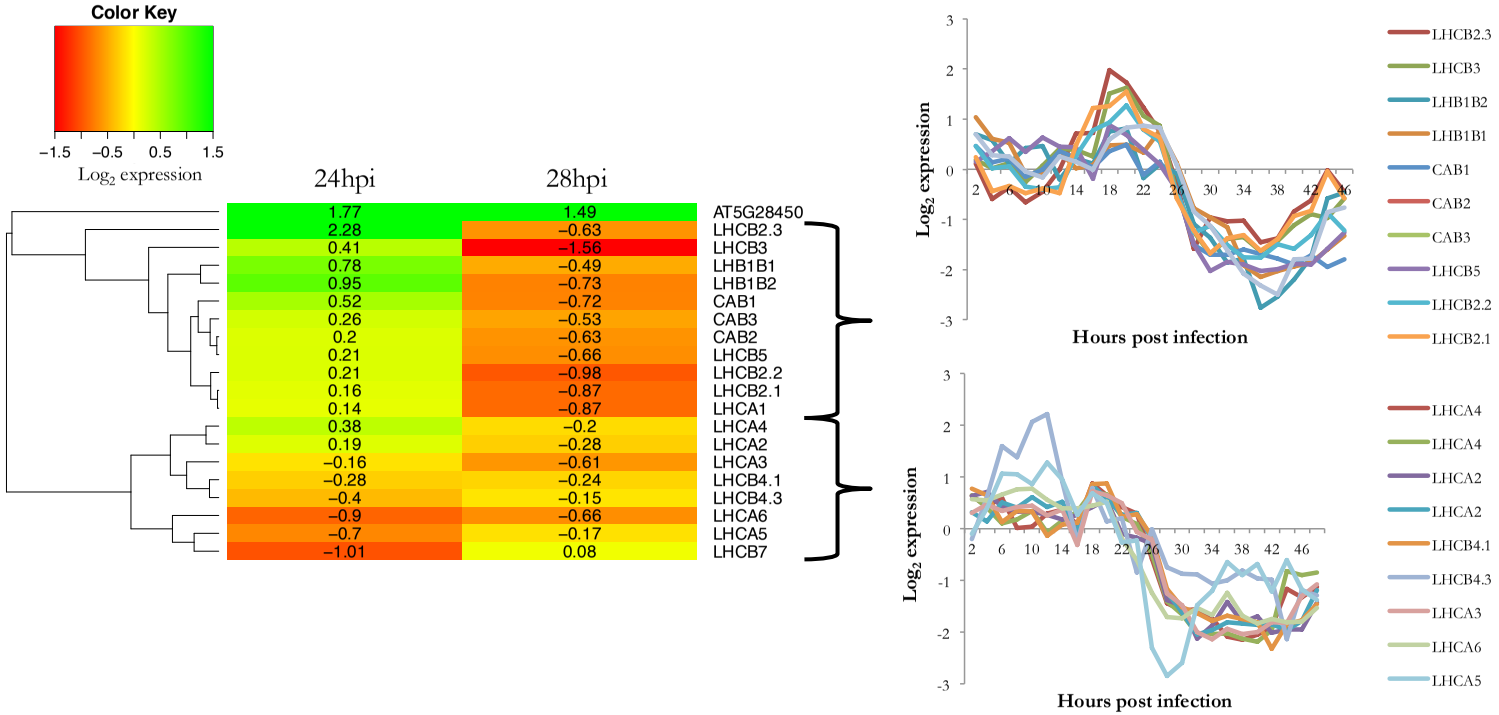


Figure 3.16: **Expression of LHCB genes during Botrytis infection in Col 0 and *anac092-1*** Expression of genes associated with Light-Harvesting Complex GO term in *anac092-1* at 24 and 28 hours post infection, expressed as $\log_2$fold change in expression from Col 0. Genes are clustered by hierarchical clustering with their expression levels. In addition, PRESTA time series expression data is shown for these genes during *Botrytis cinerea* infection, displaying the sudden decrease in expression associated with downregulation of photosynthetic genes.

'Light-harvesting complex' (GO ID: 0030076) were retrieved from TAIR10 annotations. In total, 27 gene models associated with this GO ID, mostly members of LIGHT HARVESTING COMPLEX BINDING protein family. During Botrytis infection, expression of the LHCB genes increases briefly, before decreasing dramatically during infection by *Botrytis cinerea* (figure 3.16). This is consistent with a downregulation in photosynthetic processes observed in leaf tissue during Botrytis infection.

In *anac092-1*, it seems the expression of some LHCB genes is higher at 24 hpi and lower at 28hpi, which may indicate a delay in the expression profile seen in the PRESTA timeseries data, i.e. the curve has 'shifted' to the right. This could suggest a decrease in photosynthetic mechanisms is delayed in the *anac092-1* mutant, which leads to the absence of yellowing around the lesion site (figure 3.6). However the reduction in expression of LHCB genes at 28 hours post infection may indicate that downregulation of photosynthetic machinery is increased at this time point. Perhaps, in *anac092-1,* senescence in the area around the lesion site is delayed, but when it does occur it is accelerated.

3.3. Discussion

3.3.1. ANAC092 is a functional gene during biotic stresses

In this work, a major regulator of leaf senescence has been shown to be involved in response to *Botrytis cinerea* infection. The *ANAC092* transcript dramatically increased during infection by *Botrytis cinerea*. Transgenic lines of Arabidopsis overexpressing *ANAC092* demonstrated increased disease susceptibility, characterised by high chlorosis and increased lesion spreading. Arabidopsis deficient in *ANAC092* demonstrated increased resistance to Botrytis, with reduced lesion size and chlorosis. Taken together, this indicates that ANAC092 mediated transcription may enhance disease spreading during *Botrytis cinerea* infection.

ANAC092 is a transcription factor that has been shown to induce expression of senescent related genes such as *BFN1* during senescence (Farage-Barhom *et al.*, 2008; Matallana-Ramirez *et al.*, 2013). In an *ANAC092* mutant, a subset of senescence related genes were downregulated, including the well known senescence specific gene *SAG12*. This suggests their expression in Col 0 during *Botrytis cinerea* infection is induced by ANAC092. Senescence is known to occur in the area surrounding the lesion site (Swartzberg *et al.*, 2008), therefore it is possible ANAC092 functions in promoting this senescence-like process that occurs during a *Botrytis cinerea* infection.

In addition to activation of senescence related genes, ANAC092 has been shown to interact with the transcription factors GLK1 and GLK2 (Rauf *et al.*, 2013). GLK1 and GLK2 proteins have been shown to function in development and maintenance of photosynthetic machinery (Waters *et al.*, 2009). The ANAC092 protein interacts with GLK1 or GLK2 and inhibits their activity, thus repressing growth and development (Rauf *et al.*, 2013). GLK1 and 2 have also been shown to function in response to *Botrytis cinerea* infection, with overexpressors showing enhanced resistance to *Botrytis cinerea* and GLK1/2 double mutants showing enhanced susceptibility (Murmu *et al.*, 2013). A number of genes were upregulated in the *anac092-1* plants during *Botrytis cinerea* infection, possibly indicating their expression is promoted by GLK1/2 in normal mature leaf tissue, but is repressed by the ANAC092 mediated repression of GLK1/2 during *Botrytis cinere*a infection. This suggests an alternative mechanism by which ANAC092 may be involved in response to *Botrytis cinerea* infection, as it may control the switch from growth and development to stress response by inactivating photosynthetic genes.

A number of genes were differentially expressed in *anac092-1* during *Botrytis cinerea* infection, many of which correlated with the genes differentially expressed in protoplasts overexpressing *ANAC092* and an inducible overexpressor of *ANAC092.* These may indicate direct targets of ANAC092. Many of these appear to have a role in senescence and/or *Botrytis cinerea* infection, therefore they may represent the mechanisms by which ANAC092 regulates senescence and response to Botrytis.

In addition, a number of LHCB genes, which are involved in photosynthetic ma-

chinery, were affected by manipulation of *ANAC092* expression. The precise role ANAC092 has on their expression level is unclear, however it seems likely that perturbation in *ANAC092* expression levels alters the rate of photosynthesis decline that occurs during *Botrytis cinerea* infection.

3.3.2. ANAC092 promotes the spread of a *Botrytis cinerea* lesion on Arabidopsis leaves

One key question that has not been answered is whether the induction of *ANAC092* expression is a component of the response to Botrytis or whether Botrytis exploits *ANAC092* to enhance the infection. *Botrytis cinerea* is known to induce cell-death in the area immediately surrounding the infection site by exploiting the hypersensitive response (HR; Govrin & Levine, 2000; Govrin *et al.*, 2006), which is normally used to contain biotrophic infections (Yu *et al.*, 1998; Nimchuk *et al.*, 2003), therefore it is known that Botrytis is capable of exploiting host pathways. However, a direct link between Botrytis infection and *ANAC092* induction has not been detected.

ANAC092 expression is known to be responsive to a signalling cascade triggered by EIN2 (Kim *et al.*, 2009). In turn, EIN2 is activated by binding to the ethylene receptor ETR in the presence of ethylene (Guzmán & Ecker, 1990; Ji & Guo, 2013), but also functions in ABA signalling (Wang *et al.*, 2007). *ANAC092* transcript dramatically increases upon ethylene treatment (Kim *et al.*, 2009) and appears to function in ABA induced senescence (Kim *et al.*, 2011). *Botrytis cinerea* produces both ABA (Siewers *et al.*, 2004, 2006) and ethylene (Qadir *et al.*, 1997; Lloyd *et al.*, 2011) during infection, which may be responsible for the increase in *ANAC092* expression. In this study, expression of *ANAC092* was diminished, but not abolished in an ethylene insensitive mutant, suggesting ethylene perception is part, but not all of the reason *ANAC092* responds to Botrytis infection. This suggests transcription of *ANAC092* may be partly promoted by ethylene manufactured by Botrytis, although other mechanisms such as ROS and ABA signalling may contribute to expression.

Despite these suggestions, it is still possible that ANAC092 is used by Arabidopsis to ensure continued survival during pathogen attack. During *Botrytis cinerea* infection, senescence may be induced through ANAC092 as a recycling mechanism to withdraw macromolecules and nutrients away from the lesion site, thus preventing *Botrytis cinerea* from acquiring them. In this study, lesion size was used as a measure of phenotype, which has been previously successful, but is very simplistic. A larger lesion size indicates a larger spread of *Botrytis cinerea* infection, but if senescence has been used to move nutrients away from the lesion site, the infection may not actually be as successful. This illustrates the issue with using a single measurement such as lesion size.

3.3.3. ANAC092 appears to share a function between necrotrophs and biotrophs

Necrotrophs such as *Botrytis cinerea* physically damage the cells before absorbing the nutrients released. By comparison, biotrophs such as *Hyaloperonospora arabidopsidis* will attempt to sequester nutrients from within living cells (Panstruga, 2003; Glazebrook, 2005). In response to these differing pathogens, Arabidopsis has involved a complicated and multi-faceted respond to different organisms in different manners. Broadly, Arabidopsis will attempt to maintain cell viability in response to a necrotrophic pathogen attack through JA/ET dependent responses, while during response to biotrophs Arabidopsis will initiate a range of responses that degrade and destroy their own cells to prevent the pathogen utilising the cellular components (Glazebrook, 2005).

Therefore, since *anac092-1* was more resistant to *Botrytis cinerea*, it was expected it will be more susceptible to a pathogen with an opposing lifestyle such as *Hyaloperonospora arabidopsidis* (Coates & Beynon, 2010). But instead *ANAC092* transgenic lines demonstrated a similar phenotype in response to *Hpa* as they did to *Botrytis cinerea*, i.e. *anac092-1* was slightly more resistant to *Hpa* while the overexpressor of ANAC092 was slightly more susceptible. This may indicate that *ANAC092* upregulation is broadly effective against many forms of biotic stress. Alternatively, upregulation of *ANAC092* transcript during *Botrytis cinerea* infection is unique amongst necrotrophic organisms, therefore the resistance to *Botrytis cinerea* conferred by mutation in *ANAC092* can be considered anomalous amongst necrotrophs. Tests with a related but distinct species that do not induce senescence such as *Trichoderma harzianum* (Swartzberg *et al.*, 2008) may reveal differences in ANAC092 function.

ANAC092 has previously been seen to function in biotic stress in the context of age-related resistance, whereby it performs two functions. Firstly, it retards the age of the plant, leading to a later transition to flowering and establishment of ARR. Secondly, it negatively contributes to ARR once it is initiated. In this work *anac092-1* was demonstrated to be resistance to *Botrytis cinerea* infection, however it was only tested at one age. Susceptibility to *Botrytis cinerea* changes as the plant ages (Jarvis, 1977; Elad, 1995; Kretschmer *et al.*, 2007), therefore it is possible that *anac092-1* plants have an altered phenotype due to a delay in their biological age. Further work is needed to clarify this.

3.3.4. Other senescence regulators may show differing phenotypes during biotic stress

The breeding of 'staygreen' varieties that exhibit delayed senescence has been a key target in agriculture for many years (Thomas & Ougham, 2014). In addition to remaining photosynthetically active for longer, staygreen varieties frequently exhibit enhanced tolerance to stresses (Gregersen *et al.*, 2013). Here, Arabidopsis lacking a

functional of a major promoter of plant senescence has been demonstrated to have a resistant phenotype to multiple pathogens, in addition to its previously characterised 'staygreen' phenotype (Oh *et al.*, 1997). It is possible this work is applicable to economically important crops, where plants exhibiting delayed senescence may be resistant to pathogenic stress.

It is also important to note that ANAC092 is only one of a number of known regulators of senescence. The closely related NAC protein ANAC016 has been implicated in promoting senescence (Kim *et al.*, 2013), as has AtNAP (Guo & Gan, 2006) which is regulated by the same EIN3 transcription factor as *ANAC092* (Kim *et al.*, 2014). In other transcription factor families, WRKY53 is known to promote senescence (Miao *et al.*, 2004). Similarly, the transcription factor JUB1 has been identified to be a negative regulator of senescence through manipulation of H_2O_2 levels (Wu *et al.*, 2012a). These transcription factors, alongside other regulators, may regulate senescence and response to necrotrophic species such as Botrytis. As such, it is possible many of them may represent a cross-link in the highly interconnected stress response and senescence network.

4. Identification of Transcription Factors that Bind to the *ANAC092* Promoter

4.1. Introduction

In the previous chapter, *ANAC092* expression was shown to be upregulated in response to *Botrytis cinerea* infection, where it may have a role promoting gene expression in the vicinity of the lesion site, forming a component of the response to biotic stress. *ANAC092* is also upregulated in response to a range of stresses and conditions such as time, salt, dark, pathogenic stress and hormone treatments (Kim *et al.*, 2009, 2011; Balazadeh *et al.*, 2010a). Since *ANAC092* is upregulated in response to a wide range of stresses, it must be regulated by a number of mechanisms that trigger expression under multiple conditions. The aim of this chapter is to elucidate the regulatory mechanisms behind *ANAC092* expression levels.

During developmental senescence, expression levels of *ANAC092* are increased over time through a trifurcate feed forward mechanism composed of EIN2, EIN3 and *miR164* (Kim *et al.*, 2009; Li *et al.*, 2013; Kim *et al.*, 2014). The concurrent downregulation of *miR164* and the upregulation of *ANAC092* transcript is driven by direct interaction of *EIN3* with the promoter regions of *miR164* and *ANAC092*. In turn, *EIN3* is expressed in response to activation of *EIN2*. But this process is unlikely to be the only mechanism of regulation, as expression of *ANAC092* in ageing tissues is reduced by only ~50% in *ein3 eil1* knock-out lines (Kim *et al.*, 2014). Therefore, alternative or additional mechanisms are likely to exist for promoting *ANAC092* expression.

The EIN2-EIN3-miR164 regulatory pathway has only been studied in age-induced senescence and although this regulatory mechanism may also be active during premature senescence, this has not been defined. No determinants of *ANAC092* expression have been identified in stress conditions, such as the increase in expression observed in salt-triggered senescence (Balazadeh *et al.*, 2010a,b) or *Botrytis cinerea* response (this work). During salt-treatment, *ANAC092* transcript increases, but *miR164* levels do not decrease (Balazadeh *et al.*, 2010a), suggesting that the *miR164* system may have no role during stress conditions. Therefore, other regulatory interactions may drive expression of *ANAC092* during stress induced senescence.

Protein:DNA interactions on the DNA immediately upstream from the coding region of a gene are one of the key aspects of regulation. Transcription factors recognise specific DNA sequences at the 5' end of the gene, promoting or inhibiting the formation of the basal transcription machinery, thus promoting or repressing transcription by RNA POLYMERASE II (Smale & Kadonaga, 2003). The interactions between transcription factors and DNA are inherently relatively weak to allow the transcription factor(s) to bind or dissociate in response to stimuli. This allows transcription factors to modulate gene expression in a dynamic manner, causing dramatic increases or decreases in expression levels of the target gene depending on the conditions. Therefore, identification of these interactions is one of the most critical aspects to analysing gene regulatory networks.

The proteome environment that forms on the upstream DNA is dynamic and composition changes based on time, external stimuli, location in the plant and many other factors. The changes that occur are dependent on availability of transcription factors, post translational modifications and cellular conditions. When analysing protein:DNA interactions, initially it is important to remove much of this context and simply analyse the propensity of a protein to bind to the target DNA. As such, a large number of techniques have been developed that detect DNA binding in the absence of a native environment. These simply test the possibility of DNA:protein interactions; the user must then to identify the context specific role of that interaction in further experiments.

4.1.1. Yeast 1-hybrid as a high throughput technique for identifying protein:DNA interactions

Yeast 1-hybrid is one of the most commonly used techniques for analysing protein interactions with a DNA region of interest (Li & Herskowitz, 1993). In yeast 1-hybrid, the DNA region of interest is linked to a reporter gene, typically the *HIS3* gene from yeast, to create what is sometimes referred to as the 'bait'. The transcription factor(s) to be tested are fused to a strong activation domain, typically from yeast GAL4 (Keegan *et al.*, 1986). This is known as the 'prey'. The two constructs are used to transform a strain of yeast, auxotrophic for histidine because of a non-functional *HIS3* gene. In the absence of histidine, yeast can only grow when the prey protein is capable of binding to the bait DNA and driving expression of the *HIS3* gene. Use of the yeast cellular environment maintains many of the eukaryotic features that may be important for DNA binding, but lacks the cellular environment of a plant cell that may affect expression. Previously, yeast 1-hybrid has been used successfully in identifying potential protein:DNA interactions from many organisms, including Arabidopsis (Tran *et al.*, 2004, 2007; Zhu *et al.*, 2010; Chen *et al.*, 2010; Mitsuda *et al.*, 2010; Castrillo *et al.*, 2011; Hickman *et al.*, 2013; Kim *et al.*, 2014), suggesting yeast 1-hybrid based techniques are appropriate for identification of plant

protein:DNA interactions.

One key advantage of the yeast 1-hybrid technique is that it can be scaled up to a high-throughput method, allowing simultaneous determination of a large number of DNA:protein interactions. This is particularly pertinent to plants, as the number of transcription factors has expanded dramatically, with a predicted 1716 coded in the Arabidopsis genome (Jin *et al.*, 2014). Clearly, individual testing for this many transcription factors individually would not be feasible, therefore a system for screening a single promoter against a large number of proteins is needed.

There are various methods that allow a large number of transcription factors to be screened simultaneously in yeast 1-hybrid. One of the most straightforward is to use a cDNA library generated from a transcriptome as prey. In this technique, a transcriptome is harvested from a specific tissue (e.g. stressed roots), then used to generate a cDNA library which is screened using yeast 1-hybrid. This technique was used to identify the original NAC binding motif from a dehydration stress specific transcriptome (Tran *et al.*, 2004, 2007). The use of relevant transcriptomes allows the library to be specific to the condition or tissue that is being studied, but it will contain a high proportion of irrelevant ORFs, such as non-DNA binding proteins and housekeeping genes. Similarly, of the positive results, often the 'strongest' will out compete the weaker more subtle results. Therefore it is preferred to enrich for DNA binding proteins to assess the ability of many transcription factors to bind to DNA in an unbiased manner.

Generation of a transcription factor specific library is a huge benefit to yeast 1-hybrid screens. Recognising this need, the REGIA project (REgulatory Gene Initiative in Arabidopsis) cloned 884 Arabidopsis transcription factors (Paz-Ares & Regia Consortium, 2002), which was later expanded by addition of 288 more genes to a total of 1172 cloned transcription factors (Castrillo *et al.*, 2011). The use of a gateway cloning system (Hartley *et al.*, 2000) greatly facilitated the cloning of such a large number of genes between vectors. This library was used in yeast 1-hybrid to identify binding of *MERISTEM LAYER 1* (*AtML1*) to the 50bp promoter region of *LIPASE 1* and re-identify an interaction between the transcription factor *PHOTOLYASE 1* (*PHR1*) and a 50bp promoter fragment from the phosphate starvation gene *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*) containing a PHOSPHATE STARVATION RESPONSE REGULATOR 1 (PHR1) binding site (*P1BS*) motif, GNATATNC (Castrillo *et al.*, 2011).

A similar library was developed by Gong *et al.* (2004), consisting of 1282 Arabidopsis transcription factors. This was later expanded by Ou *et al.* (2011) to contain 1589 transcription factors and used in a dual experiment with yeast 2-hybrid. The TF library was used to screen MEDIATOR 25 (MED25) in yeast 2-hybrid for protein:protein interactions while simultaneously the library was used to screen the *PDF1.2* promoter in yeast 1-hybrid. Three ERF family transcription factors were identified to bind to the MED25 protein and the *PDF1.2* promoter, suggesting a

multi-faceted DNA-protein based signalling mechanism (Ou *et al.*, 2011).

A gene-regulatory network is believed to act at the centre of the circadian rhythm in Arabidopsis. As such, efforts to elucidate the transcription factor:DNA interactions controlling gene expression central to circadian rhythms are critical. A yeast 1-hybrid library composed of circadian-specific transcription factors was developed by Pruneda-Paz *et al.* (2009), containing 189 cloned transcription factors, representing 10% of the Arabidopsis encoded transcription factors. This library was used to detect a feedback loop composed of mutual binding of *CCA1 HIKING EXPEDITION* (*CHE*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) to one anothers promoter region to repress transcription. This reciprocal negative feedback loop are part of the underlying mechanisms that establishes the cyclical rhythms of gene transcription during a day-night cycle. In an effort to determine the role external stimuli may play in influencing the clock in Arabidopsis, the same group cloned and validated the sequence of 1956 transcription factors, representing the largest library of confirmed transcription factors in a eukaryotic species, including many from different database predictions (Pruneda-Paz *et al.*, 2014). This library was used to screen the *CCA1* promoter using a novel automated platform and a *lacZ* reporter. 58 interactions with the *CCA1* promoter fragments covering the first -772bp from the coding sequence of *CCA1*. 32 of these interactions were retested using a protoplast transactivation system, which induced an arhythmic expression of a *CCA1*::*LUC+* reporter, which normally displays a circadian rhythm, suggesting they can regulate *CCA1 in vivo* (Pruneda-Paz *et al.*, 2014). However, subsequent analysis of public microarray data revealed limited expression correlation between *CCA1* and the potential regulators, suggesting they are not expressed in similar tissues. An interaction between the transcription factor FLOWERING BHLH 1 (FBH1) in yeast 1-hybrid was confirmed to bind to a CACTAG motif on the *CCA1* promoter and suppress *CCA1* expression using ChIP and a a *CCA1*::*LUC+* reporter (Pruneda-Paz *et al.*, 2014), demonstrating the validity of using yeast 1-hybrid as a technique for determining circadian rhythm gene regulatory networks.

4.1.2. 'Pooled' Yeast 1-hybrid for large scale promoter screening

Screening of a large number of transcription factors requires extensive planning and experimental design. Screening over 1000 yeast colonies for growth by hand is simply not feasible for any time scale, therefore various strategies have to be employed. The most straightforward, but expensive, is to use robotic equipment. The experiment in Pruneda-Paz *et al.* (2014) used robotic equipment in conjunction with six 384-well plates to screen the 1956 transcription factors within the library simultaneously. However, robotic equipment is expensive and requires a level of technical expertise that may not be available. Furthermore, they require an automated system of recording results, which adds additional costs.

The PRESTA project aimed to utilise the high-throughput nature of yeast 1-hybrid to screen the promoter regions of many stress responsive genes from Arabidopsis. Therefore, a technique was developed that enabled the screening of a promoter against the transcription factor library using only a 96-well plate layout, thus enabling the simultaneous screening of many transcription factors against a single promoter.

A transcription factor library composed of 1440 transcription factors was generated by addition of a number of transcription factors to the previous library from Castrillo *et al.* (2011). The full 1440 transcription factors were inoculated in media and added to two 96-well plates, with 24 transcription factor cultures per well. This means that each well contains a combination of transcription factors, but the full transcription factor library could be contained within a single plate. There is a possibility a yeast strain containing one transcription factor could be outcompeted by others and not identified as a positive result, therefore the entire library is duplicated in another 96-well plate, with an alternative arrangement of transcription factors. The use of dual libraries also enables a simultaneous technical repeat.

Previous work by the PRESTA group has determined that large (1000bp) promoters have a high rate of false negatives, possibly because yeast promoter regions are inherently small (Dobi & Winston, 2007), therefore use of larger Arabidopsis promoters may cause position bias against protein binding results further from the transcription start site of the reporter. Therefore the use of promoter fragments smaller (400-500bp) promoter fragments has been shown to be more successful (Hickman *et al.*, 2013).

4.1.3. Aims

The aim of this chapter was to identify transcription factors capable of binding to the *ANAC092* promoter and test the potential regulation properties that each transcription factor may have on expression from the *ANAC092* promoter.

4.2. Results

4.2.1. A number of transcription factors bind to the ANAC092 promoter

4.2.1.1. Cloning of multiple overlapping promoter fragments

There is a great variety of designs of promoter fragments used in yeast 1-hybrid, from repetitive motifs (Ou *et al.*, 2011), short sequences (Tran *et al.*, 2007) or larger (1000bp) sequences (Martinez *et al.*, 2008). Dobi & Winston (2007) showed that distance from the TATAA box was inversely proportional to strength of activation in a reporter system, therefore Pruneda-Paz *et al.* (2009) developed a 'promoter hiking' system which used multiple overlapping fragments to overcome position bias. The fragments were designed to overlap to ensure no region responsible for binding is split between two fragments.

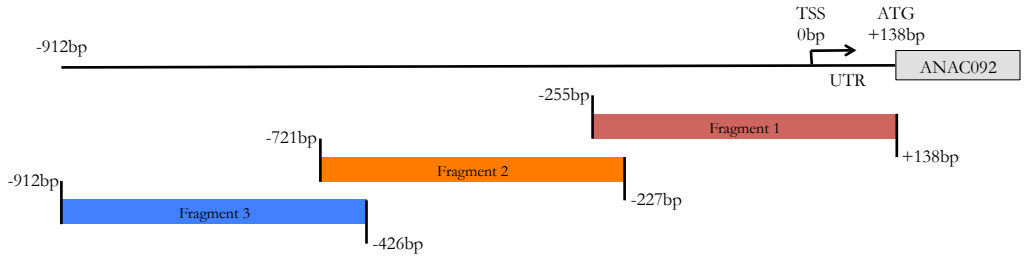


Figure 4.1: **Cloned ANAC092 promoter fragments** Diagram of the *ANAC092* 1000bp upstream region from ATG. Illustrated are the promoter fragments used for yeast 1-hybrid analysis and TSS.

Previously, the PRESTA group had optimised yeast 1-hybrid fragments to multiple 400-500bp overlapping fragments that cover 1000bp. The 1000bp promoter of *ANAC092* was retrieved from Biomart (Smedley *et al.*, 2009) and three fragments of 400-500bp were designed by Richard Hickman (see fig 4.1). Fragments were amplified from genomic DNA and cloned into pDONR.Zeo entry vector using the Gateway system. This was done to allow the promoter fragments to be used in subsequent alternative experiments.

4.2.1.2. A number of proteins bind to ANAC092 promoter fragments in a yeast 1-hybrid screen

The three promoter fragments were cloned into the pHISLEU2GW vector (created by Claire Hill, reported in Çevik *et al.*, 2012 and Hickman *et al.*, 2013). The vector contained the *HIS3* gene downstream of the promoter fragment, thus creating the necessary reporter construct. All promoter constructs were used to transform Y187 yeast (Clontech^{tm}).

The library was grown in the 2×96 well plates described previously before being

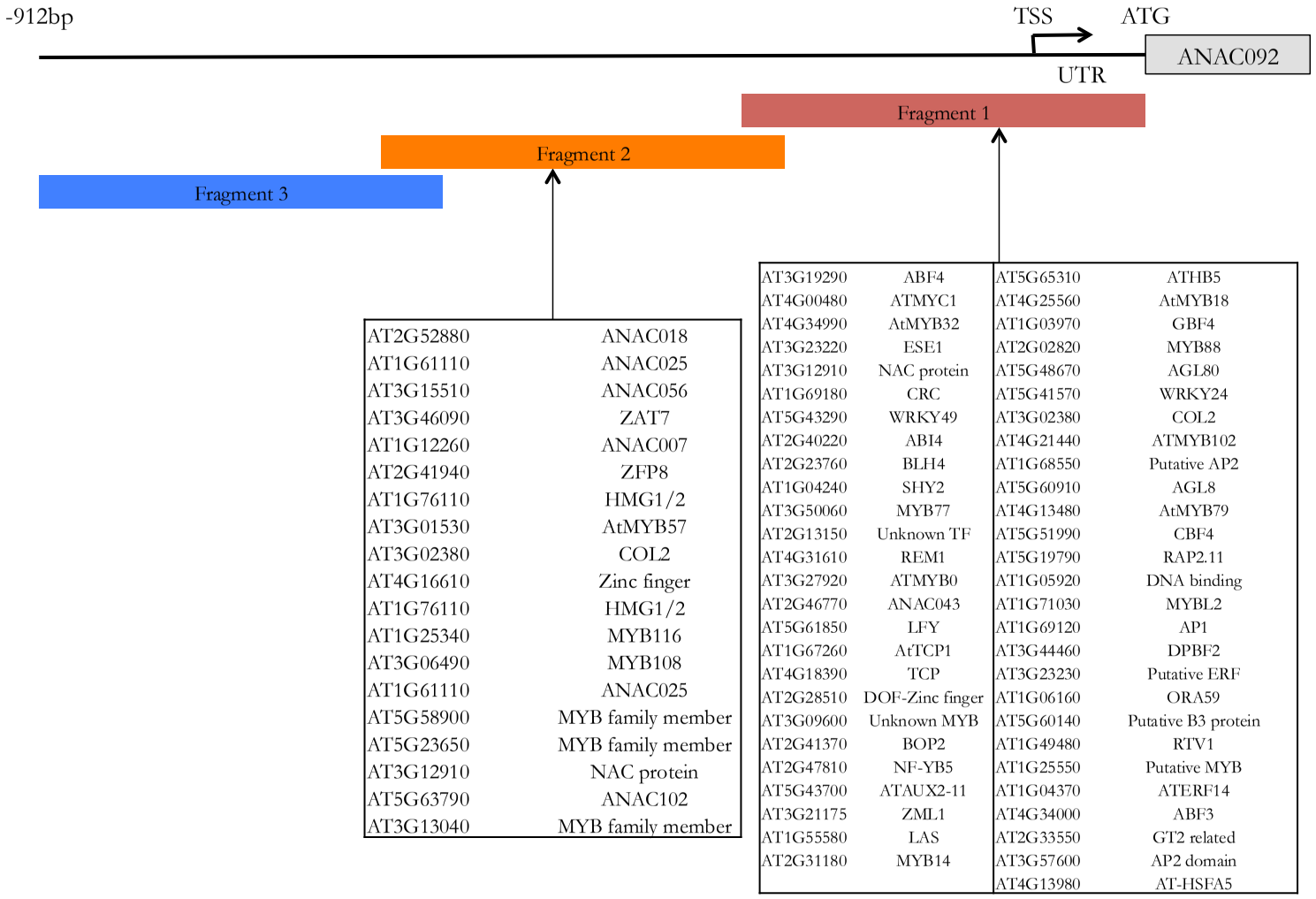


Figure 4.2: **Results from high-throughput yeast 1-hybrid analysis of the *ANAC092* promoter** Positive results from high-throughput yeast 1-hybrid screen mapped to interacting promoter region. No transcription factors appeared to bind to fragment 3.

mated on solid media with the Y187 alpha mating type containing the promoter reporter construct. Mated yeast were replica plated to selective media for diploid strains (SD minus Leucine and Tryptophan) and selective media for positive results (SD minus Leucine, Tryptophan and Histidine). Endogenous yeast proteins can sometimes activate the promoter, generating a lawn of nonselective growth. In these cases, the HIS3 inhibitor 3-aminotriazole (3-AT) was used as a competitive inhibitor at a range of concentrations. The concentration of 3-AT was adjusted to an appropriate level based on the promoter fragment being tested. Results were positive if there was growth above background levels on the selective media. Background levels for each promoter region were determined visually, looking for no or minimal growth per mating spot location. Subsequently the transcription factor gene was amplified from colonies using primers specific to the pDEST22 (transcription factor containing) vector using PCR. These PCR products were sequenced using the one of the primers for identification of the interacting transcription factor.

A number of proteins were found to bind to the *ANAC092* promoter. Promoter fragment 3 did not show any positive results over background rate at any concentration of 3-AT, while 19 transcription factors bound to fragment 2. A large number of proteins bound to fragment 1, the closest fragment to the coding sequence of ANAC092 on the Arabidopsis genome (figure 4.2).

4.2.1.3. Confirmation of observed results using pairwise yeast 1-hybrid analysis

High-throughput yeast 1-hybrid identified a number of potential protein interactions with the *ANAC092* promoter. To verify these, they were repeated individually in a one-on-one combination. Plasmids containing the transcription factor were retrieved from bacterial stocks, before being sequenced to confirm no mutations were present in the coding sequence. These were used to transform AH109 yeast and arranged in a custom library, with GFP and an empty vector was included as a negative control. The screen was repeated as previously, but the transcription factor clone was not sequenced as each well only contained a single clone. This meant that each transcription factor was tested against each promoter fragment individually, which is referred to as a 'pairwise' screen (figure 4.6). This was repeated three times, overall positive results were taken as two positive results in individual screens.

The repeated pairwise yeast 1-hybrid confirmed a number of the interactions. For fragment 2, several MYB and NAC protein interactions were confirmed as repeatable results. For fragment 1, only 4 observations were repeated successfully. It is possible many of the previous results were an example of autoactivation, that were identified as false positives because of the lack of a negative control in the high-throughput screen. In the pairwise screen, the inclusion of GFP and an empty pDEST^{tm}22 allow determination of a 'true' negative, which reduced the incidence of false positives (figure 4.3).

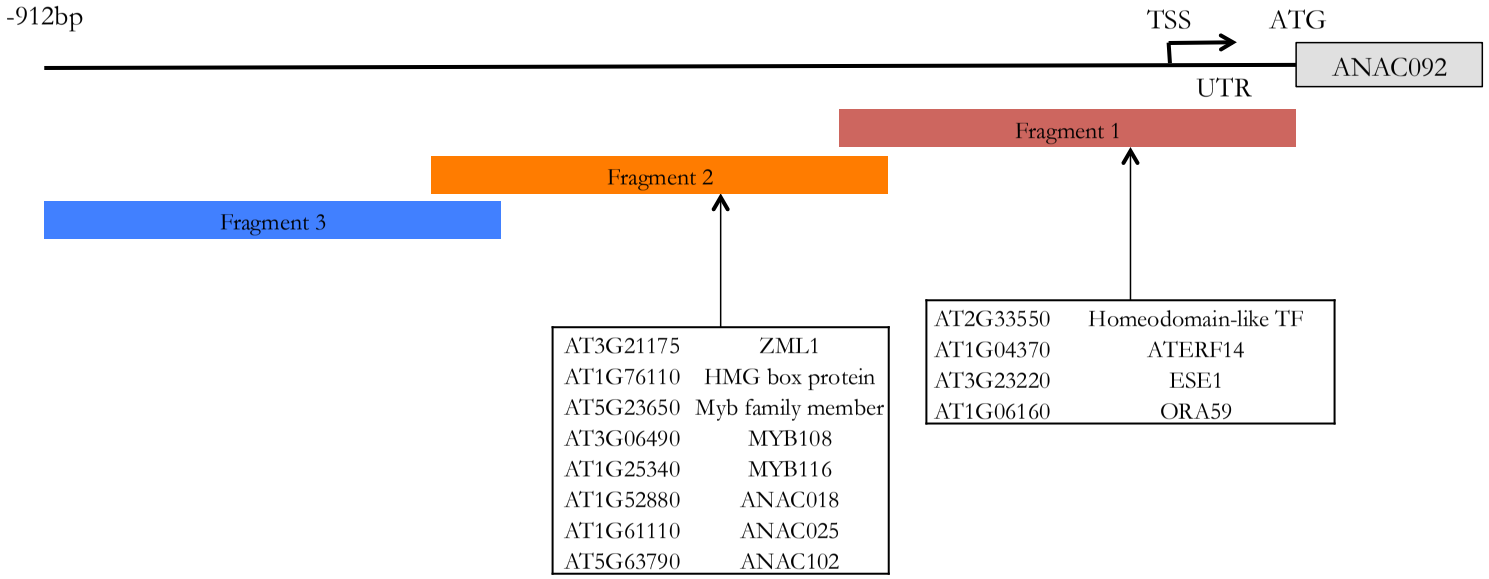


Figure 4.3: **Results from pairwise yeast 1-hybrid of the *ANAC092* promoter** Positive results in pairwise yeast 1-hybrid mapped to cloned promoter fragments of *ANAC092*.

4.2.1.4. 'Promoter chopping' identifies smaller regions that may be responsible for protein binding

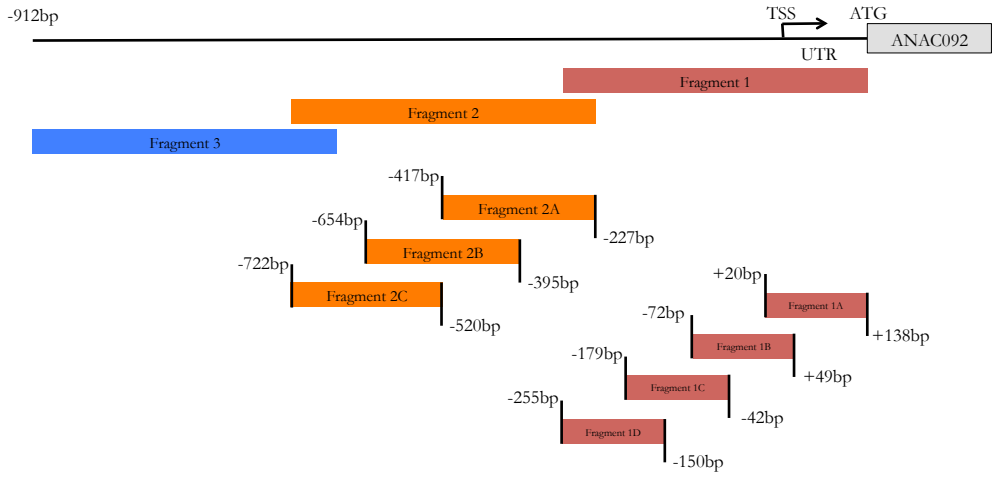


Figure 4.4: **'Chopped' promoters for yeast 1-hybrid analysis** Diagram of promoter fragments cloned into smaller overlapping fragments for use in promoter 'chopping' experiments, showing start and end relative to ATG of *ANAC092*.

The use of overlapping promoters was intended to reduce the rate of false negatives, but had the added benefit of reducing the possible area that the proteins bound to. If a protein bound to two promoter fragments that shared a small region, it can be suggested that the proteins are bound to that overlapping region. Transcription factor recognition site identification is a critical area for analysis of gene regulatory networks, therefore promoter fragments that showed positive results were divided into multiple overlapping fragments. Because fragment 1 had shown so many positive interactions in the initial screen, it was decided to divide it into four overlapping fragments of ~100bp, while fragment 2 was divided into 3 fragments of around ~200bp (figure 4.4).

The 'chopped' promoter fragments were screened against each positive result again, resulting in a number of positive interactions. As before, interactions that were reproduced at least two of three times were classed as positive results. These results are illustrated in figure 4.5. A number of transcription factors bound to the reduced size promoter fragments. For example, ZML1 was able to bind to fragment 2A and 2B, indicating they shared a region ZML1 can recognise (figure 4.7).

4.2.1.5. Summary of results from yeast 1-hybrid analysis of the *ANAC092* promoter

After verification with pairwise yeast 1-hybrid, 13 transcription factors were shown to bind reproducibly to the promoter region of *ANAC092*. These included, but were not exclusive to, members of the ERF, NAC and MYB transcription factor families.

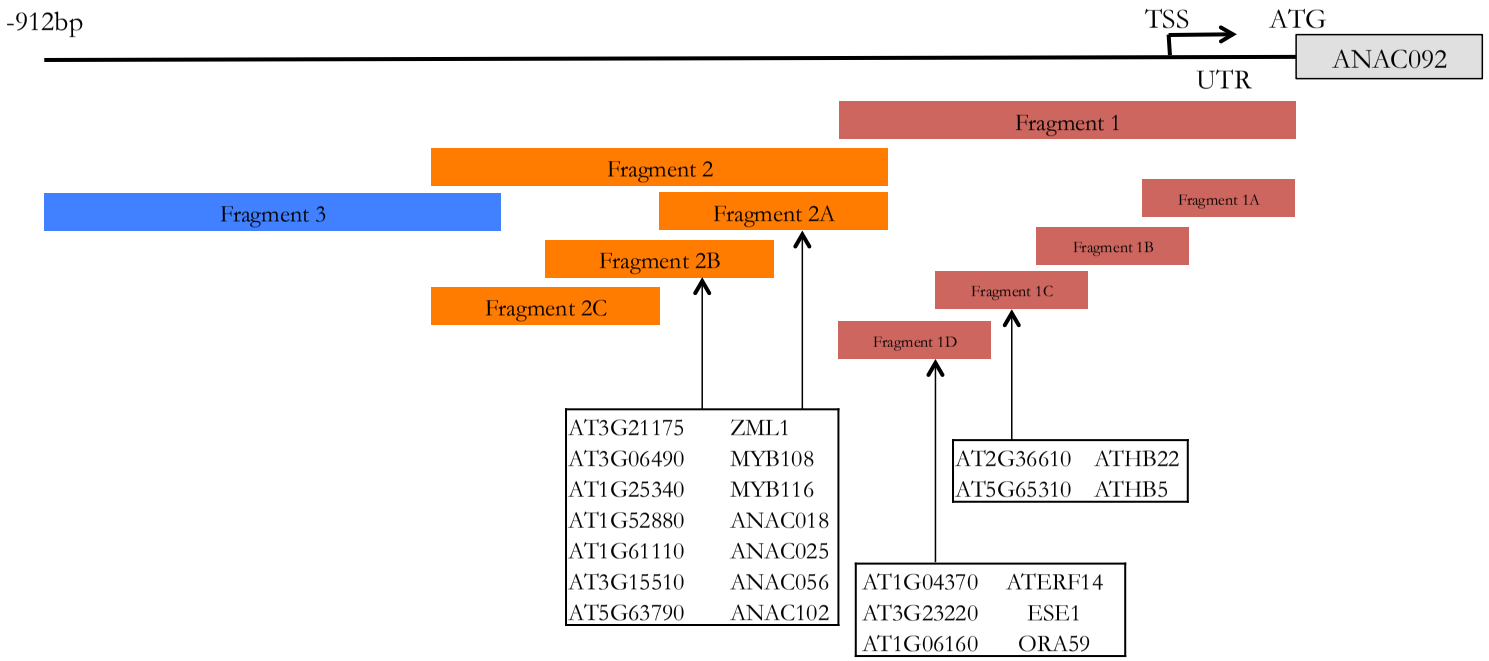


Figure 4.5: **Results from pairwise yeast 1-hybrid screening of 'chopped' yeast 1-hybrid promoter** Positive results in pairwise yeast 1-hybrid mapped to cloned 'chopped' promoter fragments of *ANAC092*.

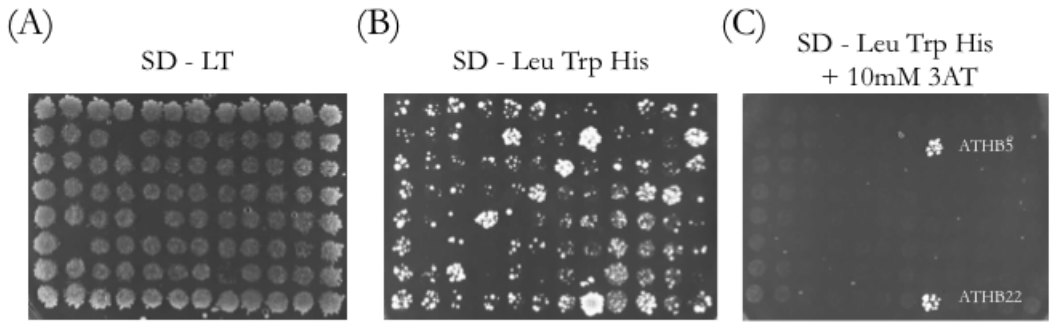


Figure 4.6: **Pairwise yeast 1-hybrid analysis of fragment 1C** Promoter region 1C tested against the pairwise library of transcription factors. (A) SD - Leu Trp is media lacking Leucine and Tryptophan for plasmid selection, but contains Histidine, allowing growth of any yeast that has successfully mated. (B) SD - Leu Trp His lacks Histidine and is selective for positive results. In this example, there is a high rate of autoactivation of the promoter region by endogenous yeast transcription factors, therefore the *HIS3* inhibitor 3-aminotriazole is used at a concentration of 10mM to restrict background growth revealing positive colonies (C). These were identified as *ATHB5* and *ATHB22*.

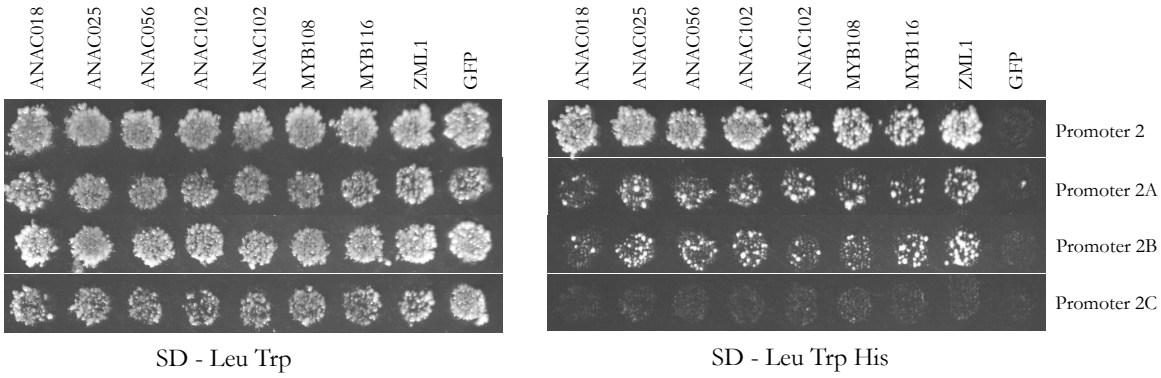


Figure 4.7: **Yeast 1-hybrid analysis of 'chopped' promoter fragment 2** Promoter region 2, 2A, 2B and 2C tested individually against 8 transcription factors. The left image is non-selective SD - Leu Trp, the right image SD - Leu Trp His, which shows positive results for the left and middle fragment in addition to the full 400bp promoter.

Members of the same transcription factor family bound to the same promoter fragments of the *ANAC092* promoter. For example, ATHB5 and ATHB22 bound to the promoter fragments covering the 317 - 180bp region upstream of the *ANAC092* ATG. Both of these proteins are members of the HD-Zip family, a plant specific family of transcription factors that have been shown to recognise an inverted repeat sequence CAATnATTG (Johannesson *et al.*, 2001). This sequence is present within the *ANAC092* promoter region at the point where ATHB5 and ATHB22 bind (figure 4.9).

Similarly, four NAC proteins bound to several promoter fragments that all share

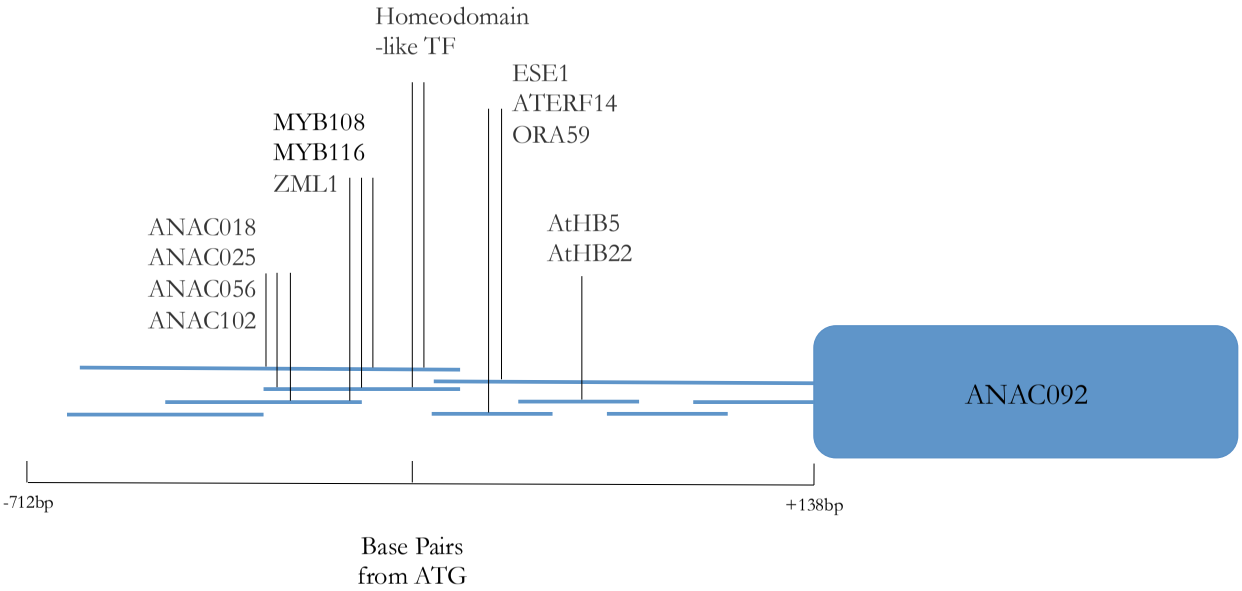


Figure 4.8: **Final results from yeast 1-hybrid analysis of the ANAC092 promoter region** Diagram of transcription factors binding to the promoter of *ANAC092* as identified by pairwise yeast 1-hybrid. Promoter fragments are illustrated as horizontal blue lines, with the ATG of *ANAC092* is on the right side. Transcription factors are illustrated in black and vertical lines indicate which promoter fragment they bind to. Transcription factors are grouped by transcription factor families. Multiple vertical lines indicates a single transcription factor was capable of binding to more then one promoter fragment.

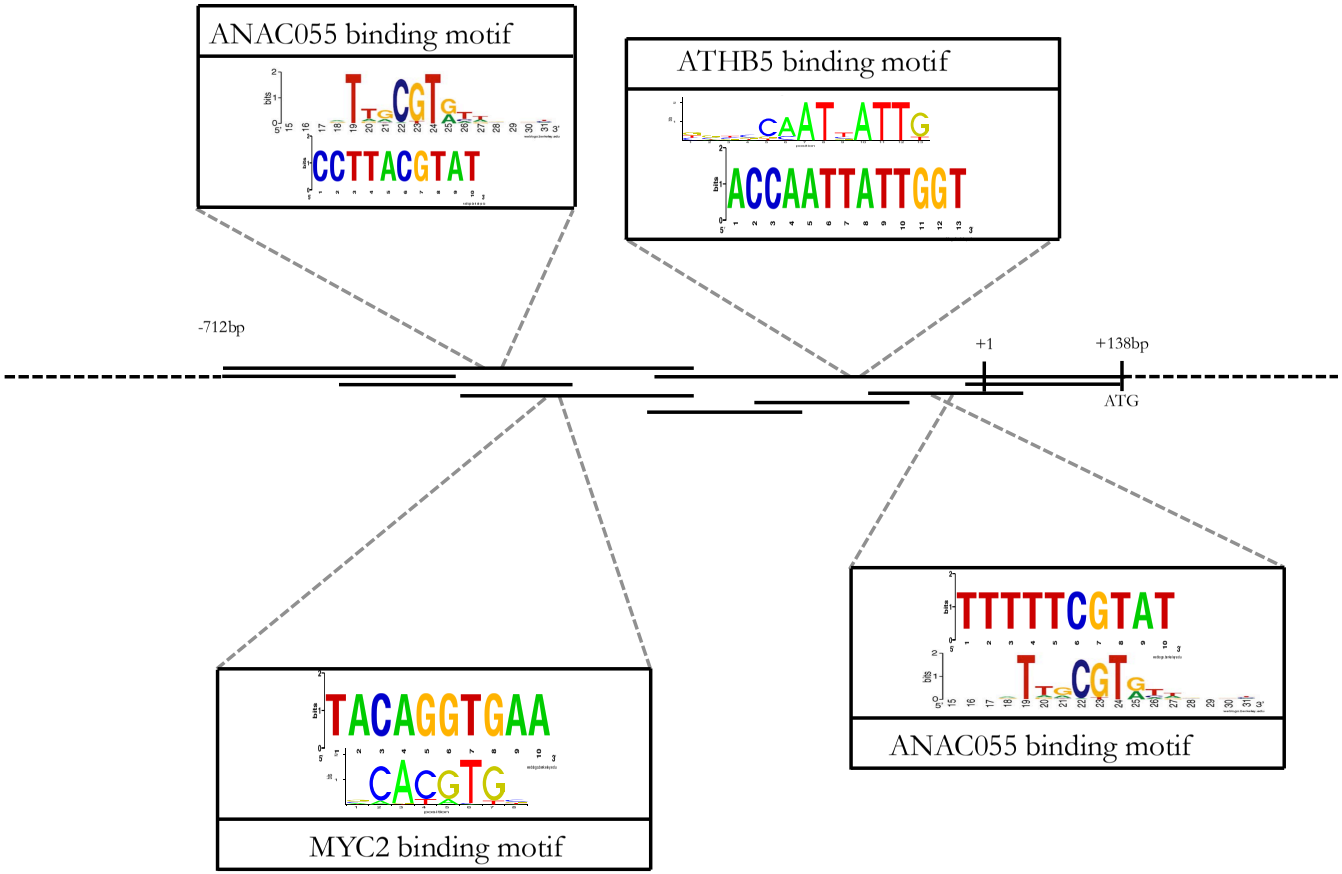


Figure 4.9: **Location of relevant transcription factor motifs on *ANAC092* promoter region** The *ANAC092* promoter fragments with relevant transcription factor binding sites annotated. ANAC055 recognition motif is from Olsen *et al.* (2005a), ATHB5 recognition motif is from Johannesson *et al.* (2001) and MYC2 recognition motif is from Godoy *et al.* (2011). Transcription factor motifs are mapped to approximate location on promoter and the sequence of the *ANAC092* promoter region is shown alongside the relevant motif. The 3' *ANAC055* motif is shown in reverse complement.

a region 600-700bp upstream from the ATG of *ANAC092*. These four NAC proteins are closely related on the phylogenetic tree of NACs and therefore it is likely they recognise a similar binding motif (Ooka *et al.*, 2003; Jensen *et al.*, 2010; Lindemose *et al.*, 2014). The binding motif for ANAC018, ANAC056, ANAC025 and ANAC102 has not been determined, but the binding sites for other NAC proteins such as ANAC092 and ANAC019 are known (originally research in Olsen *et al.* 2005a and reviewed recently in Jensen & Skriver 2014). Many NAC recognition sites contain the consensus sequence CGT, with many recognising TTnCGT as a full sequence (Lindemose *et al.*, 2014). This motif is located twice in the full 1000bp *ANAC092* promoter (figure 4.9). One of these motifs is located in the promoter region where the four NAC proteins bound in yeast 1-hybrid, suggesting the NAC proteins can recognise it and form an interaction with the DNA. The second NAC motif located is located in the first 250bp region upstream from the ANAC092 coding region but faces in the opposite direction to the other motif. No NAC proteins were observed to bind to a promoter region covering this motif in this yeast 1-hybrid experiment, suggesting direction of the NAC recognition motif influences NAC protein binding.

A number of ERF proteins such as ORA59, ATERF14 and ESE1 bound to the *ANAC092* promoter. ORA59 has previously been shown to bind to a GCCGCC motif ('GCC box') in the *PDF1.2* promoter (Zarei *et al.*, 2011), but a GCC box is not located on the promoter region of *ANAC092* that has been screened. In fact, the first GCC box is located 2691bp upstream from the *ANAC092* ATG on Arabidopsis. In Oñate-Sánchez *et al.* (2007), three ethylene responsive genes, *ERF1*, *ATERF2* and *ATERF15*, were diminished in expression in *aterf14* mutants following ethylene treatment. *ERF1*, *ATERF2* and *ATERF15* do not contain GCC boxes in their promoters either, leading the authors to suggest ATERF14 can regulate these genes through an alternative binding motif or act through an intermediary. It is possible that ATERF14 can recognise a binding motif other then the GCC box in yeast 1-hybrid which is located on the *ANAC092* promoter.

Several transcription factors from the same family were capable of binding to the same regions of the *ANAC092* promoter. This suggests that the conserved DNA-binding domains shared between related transcription factors recognise similar regions of DNA and the *ANAC092* promoter encodes a single recognition motif for these related proteins. We can envisage a scenario where certain members of the transcription factor family perform different functions in regulating *ANAC092* and bind to the promoter region of *ANAC092* under different stress conditions.

4.2.2. A number of transcription factors can drive expression of a reporter enzyme from the *ANAC092* promoter in a protoplast system

Although effective for showing DNA-binding interactions, yeast 1-hybrid is a yeast based technique which lacks other plant proteins, post translational modifications and other *in vivo* conditions that may be important for transcription factor activity. Furthermore, yeast 1-hybrid links the cloned transcription factor to a strong activation domain (from yeast *GAL4*) which overcomes any intrinsic activation ability of the transcription factor itself. Finally, transcription factors do not act alone and in some instances have been identified to only promote transcription upon heterodimerisation with another protein (Fan & Dong, 2002; Tran *et al.*, 2007) which will not be present in yeast. Therefore, to study the affect of transcription factors on the *ANAC092* promoter in a more natural environment, a plant protoplast system was employed.

Protoplasts can be generated from leaf mesophyll cells that are separated from the body of the organ and stripped of their cellulose cell wall. This generates a transient cell culture of plant cells in suspension that can be transfected with different constructs for study. Because of their ease of use and their plant intracellular environment, they are commonly used to validate results detected in *in vitro* techniques. Previously, protoplasts have been used successfully in validating transcription factor interactions identified by yeast 1-hybrid in *Arabidopsis* (Tran *et al.*, 2004, 2007; Pruneda-Paz *et al.*, 2014).

The promoter regions of *ANAC092* that had been used in the yeast 1-hybrid were cloned into a gateway compatible GWGUS1 vector (created by Dr. Justyna Prusinska). This vector linked the promoter region to a minimal 35S promoter and ß-glucuronidase as a reporter. This construct was used to transform protoplasts together with a plasmid containing the transcription factor expressed from a double 35S promoter construct. Unlike yeast or bacterial cells, transformed protoplasts can not be selected using a resistance marker or auxotrophic requirements. Therefore, a vector containing luciferase expressed from the same double 35S promoter construct was used to monitor transformation efficiency. Proteins were extracted from protoplasts before a MUG assay was performed using determine transactivation of the GUS reporter. A luciferase assay of these proteins was used for transformation control (figure 4.10)

A plasmid expressing the yeast GAL4 DNA-binding domain (GalDB) by a double 35S promoter construct was used as a negative control due to its low intrinsic activation ability. This modified form of GAL4 is lacking the activation domain, but is still capable of binding to DNA (Keegan *et al.*, 1986). GALDB should not have any effect on transcription and therefore the reporter gene will be expressed at a background rate caused by the promoter alone.

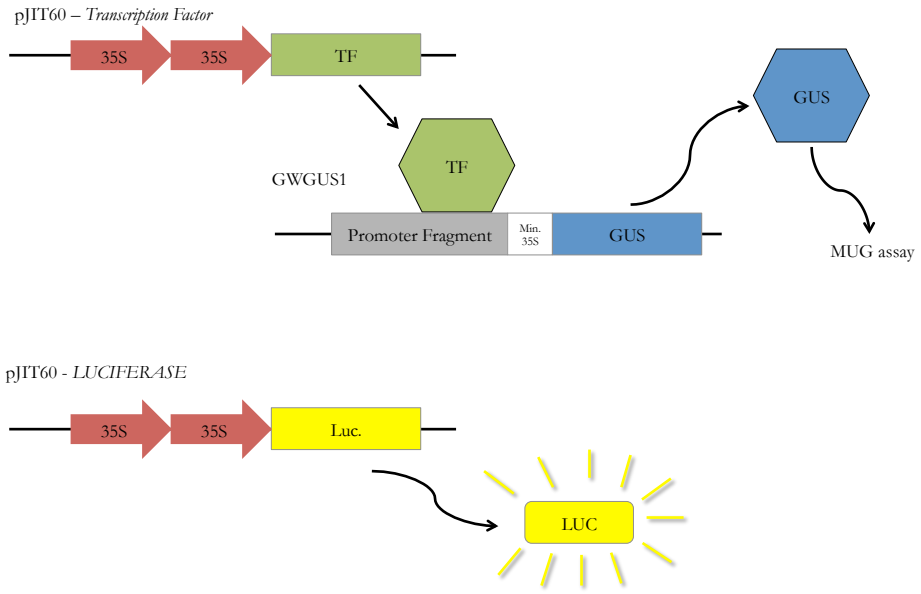


Figure 4.10: **Diagram of protoplast transcription assay** The transcription factor is expressed from two CaMV35S promoters on the pJIT60 vector (Guerineau *et al.*, 1992). The ß-GLUCURONIDASE (GUS) enzyme is expressed from the cloned promoter fragment and minimal CaMV35S (which has a low background transcription rate) on a GWGUS1 vector constructed by Justyna Prusinska. Expression of the GUS reporter gene is detected by a MUG assay. Luciferase is expressed from the same double CaMV35S promoter used for the transcription factor. This is used as an assessment of transformation efficiency.

4.2.2.1. MYB108 and MYB116 transcription factors appear to drive expression of the GUS reporter from the *ANAC092* promoter fragment

Transformation with vectors expressing the MYB genes *MYB108* and *MYB116* caused strong expression of the GUS reporter compared to the GalDB control (see figure 4.11B). This suggests they are capable of driving expression of the *GUS* reporter from the promoter fragment of *ANAC092*. This correlates with the yeast 1-hybrid data, which showed MYB proteins could bind to fragment 2. Therefore it can be suggested MYB108 and MYB116 are capable of binding to the fragment 2 promoter region of *ANAC092* and driving expression.

4.2.2.2. ERFs do not appear to drive expression of the GUS reporter from the *ANAC092* promoter fragment

Expressing *ATERF14* and *ESE1* in protoplasts did not cause the reporter gene to be upregulated, as determined by the MUG assay. However expression of *ORA59* did induce some (but not significant) upregulation ($p = 0.06$, figure 4.11C). This implies ORA59 may be able to drive transcription from the promoter fragment of *ANAC092,* however it may have weak transcriptional activity. ESE1 and ATERF14 did not appear to drive expression of the reporter, which may mean they do not bind

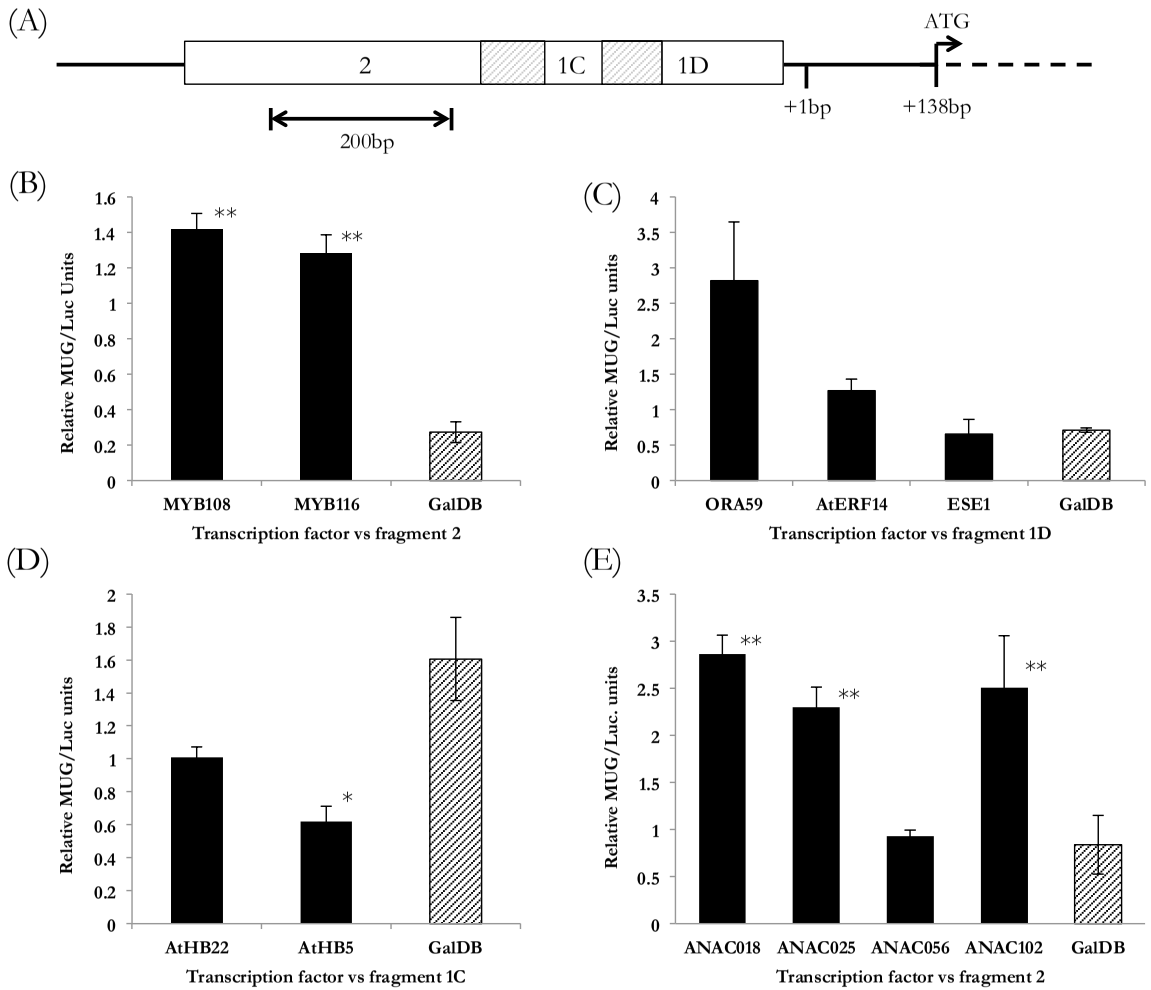


Figure 4.11: **Results of protoplast transactivation assay for *ANAC092* promoter region** (A) Illustration of promoter fragments used in this protoplast assay. Shaded area indicates overlap of promoter fragments. (B - E) Expression of promoter GUS fusions in Arabidopsis mesophyll protoplasts. The protoplasts were co-transfected with three constructs containing $p._{ANAC092}$:GUS, 2×35S:*TF* and 2×35S:*LUC+* The MUG assay was used as a determinant of transient expression by the transcription factor, while the luciferase assay was used as a control of transfection. GalDB was used as a negative control of reporter expression. * $p<0.05$ ** $p<0.01$ in Student's two-tailed unpaired t-test compared to respective GalDB. Error bars are standard error of the mean for 3 biological replicates (three independent transformations of the same protoplast culture).

to the promoter region in protoplasts, or they are unable to induce transcription from the promoter fragment. Perhaps, ERF proteins require additional proteins or cofactors to act as transcriptional activators in a protoplast system.

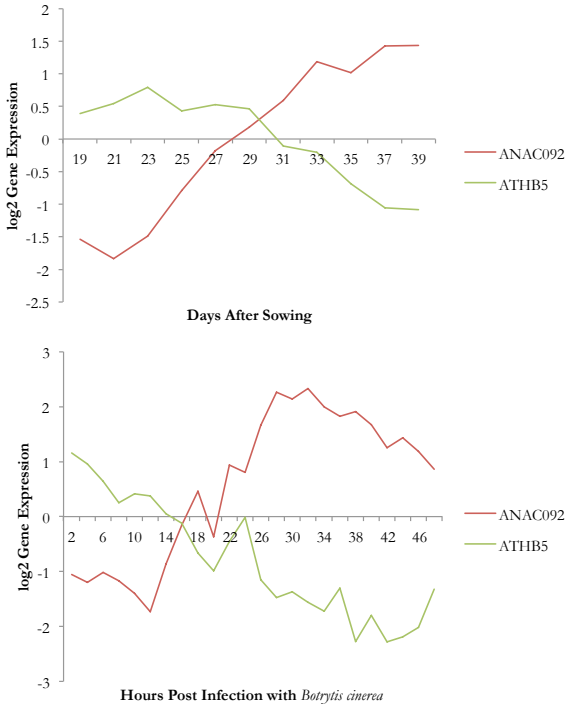


Figure 4.12: **Expression of *ANAC092* and *ATHB5* during developmental senescence and *Botrytis cinerea* infection** Expression of *ANAC092* and *ATHB5* during age-induced senescence (top; Breeze *et al.*, 2011) and *Botrytis cinerea* infection (bottom; Windram *et al.*, 2012), as determined from the PRESTA timeseries data. Expression is displayed as normalised $\log_2$ expression with a mean of 0.

4.2.2.3. HBs may be negative regulators of expression from the *ANAC092* promoter

Transformation of protoplasts with vectors expressing *AtHB5* or *AtHB22* did not show any increase from the MUG assay compared to the GalDB control (figure 4.11D), despite members of the ATHB family containing strong intrinsic activation domains (Meijer *et al.*, 2000). In fact, there was a small decrease in MUG reporter compared to the GalDB control suggesting that they may function as repressors of transcription in this assay. If the expression of *ATHB5* and *ANAC092* in the PRESTA timeseries data is compared, it seems *ANAC092* expression is negatively correlated with *ATHB5* expression during age-induced senescence and *Botrytis cinerea* infection (figure 4.12). This means that during developmental senescence and Botrytis infection ATHB5 levels decrease which occurs concurrently with an increase in the transcription of *ANAC092*. This supports the hypothesis that ATHB5 is a

negative regulator of *ANAC092* transcription.

Expression of *ATHB22* appears to not change significantly over the timeseries, suggesting it may not have a dynamic role in affecting *ANAC092* expression. This could mean it does not 'switch' *ANAC092* on or off, but instead remains constant. When combined with the protoplast transactivation data, this may suggest ATHB22 does not promote or repress transcription of *ANAC092*.

It is important to note this is not an assay for negative regulation. An appropriate assay for negative regulation would use a strong positive signal, such as a reporter gene under constitutive expression, which would be repressed upon addition of the putative repressor. In the transactivation assay, the background rate of GUS expression is low, therefore any repression might be a fluctuation about normal levels.

4.2.2.4. Three NAC transcription factors drive expression of a GUS reporter from the ANAC092 promoter fragment

Expression of *ANAC018*, *ANAC025* and *ANAC102* in the protoplast containing GUS expressed from fragment 2 induced significant upregulation of the reporter gene (figure 4.11E). *ANAC056* also appeared to induce high expression of the GUS reporter gene, however it was repeatedly noticed that the luciferase transfection control was significantly higher in the *ANAC056* transformed protoplasts then in the GalDB sample. Since the reading is proportional to the luciferase reading used as a transfection control, *ANAC056* had consistently lower results than other NAC proteins even though the MUG reading was very high. Therefore, it was proposed that *ANAC056* might also drive expression of the luciferase reporter, perhaps by also binding to the upstream CaMV35S promoter of the *LUCIFERASE* gene.

To test this, it was decided to determine transactivation without the use of the luciferase transfection control. This was done by developing a protocol from Wehner *et al.* (2011) that would allow analysis of 96 protoplast transactivation assays at the same time. In the original research, this protocol had been used to test the influence of 96 ERF transcription factors on two promoters (Wehner *et al.*, 2011). However, instead of testing a large number of transcription factors, here the high-throughput protocol was used to enable 16 biological repeats for each transcription factor with the promoter region. In principle, the larger number of repetitions should remove effects caused by transformation variation within the samples, assuming all samples transform equally. This meant a result could be obtained directly from the MUG readings compared between samples.

All four NAC proteins appeared to be able to drive expression of the GUS reporter enzyme, showing significantly higher MUG readings then the GALDB control (figure 4.13). As with the low throughput experiments that included a luciferase transfection control, ANAC018 and ANAC102 addition resulted in the strongest expression while ANAC056 induced expression of the GUS reporter at a lower level. This suggests

that all four NACs are capable of binding to the promoter region of *ANAC092* and have the ability to drive transcription. This implies that they can drive expression upon binding *in vivo* and cause *ANAC092* to be upregulated.

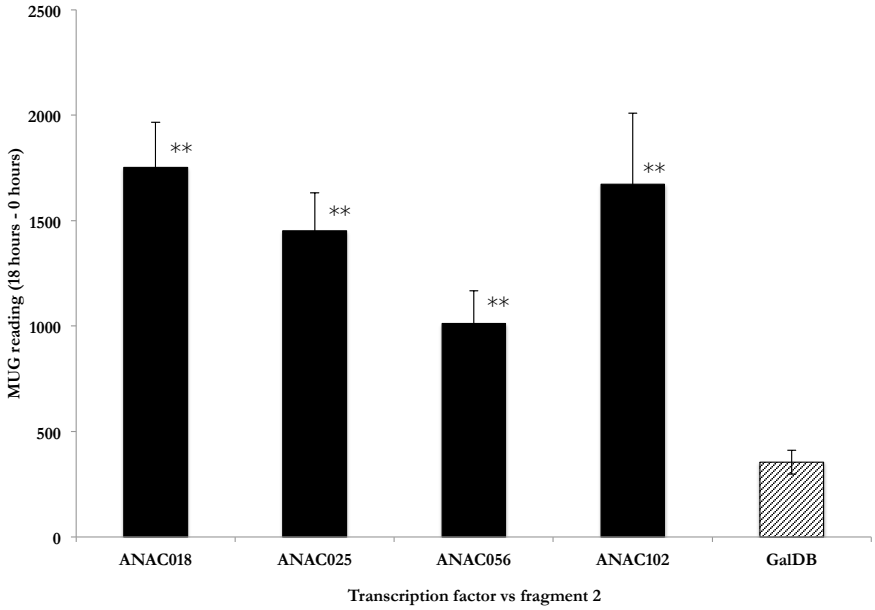


Figure 4.13: **High-throughput protoplast transactivation assay of NAC proteins against fragment 2 of the *ANAC092* promoter** MUG assay conducted on proteins extracted from protoplasts transfected with ß-glucuronidase expressed from fragment 2 promoter region of *ANAC092* over 18 hours. Each sample is cotransfected with a transcription factor or GalDB. Error bars represent SEM (N=16), ** indicates $p<0.01$ on a Student's t-test.

4.3. Discussion

ANAC092 was originally identified as a gene that was expressed in response to developmental senescence (Oh *et al.*, 1997) and later studies illustrated it was also expressed in response to a number of conditions such as salt stress, perpetual darkness, mechanical wounding and hormone treatment (He *et al.*, 2005; Balazadeh *et al.*, 2010a,b). As a highly responsive gene whose mutants show a strong phenotypic effect, regulation of the expression of this gene must be tightly controlled to ensure it is expressed only at the correct time in the appropriate tissue. While some facets of *ANAC092* regulation have been identified (Kim *et al.*, 2009; Li *et al.*, 2013), others remain to be elucidated.

Use of a high-throughput yeast 1-hybrid technique combined with multiple overlapping promoter fragments allowed identification of proteins that have the potential to bind to the DNA immediately upstream of the *ANAC092* promoter region. This analysis has revealed a number of transcription factors that could function in the regulation of *ANAC092* during one or more stresses. However, use of promoter fusions has a number of limitations. The *GUS* and *HIS3* reporter constructs used in these techniques rely on all transcription factor recruitment mechanisms being implicit in the DNA sequence, ignoring the potential role of regulation by DNA secondary structure or chromatin remodelling that have been implicated in stress response (reviewed in Chinnusamy & Zhu 2009). Similarly, yeast 1-hybrid is an *in vitro* technique that will have a high rate of false negatives due to the differences between the plant and yeast cells that may lack post translational modifications and auxiliary proteins. Also, the addition of a strong activation domain may inhibit DNA-binding. *EIN3* has been shown to bind to the *ANAC092* promoter through ChIP-PCR and an alternative form of yeast 1-hybrid (Kim *et al.*, 2014), however this was not observed in this experiment, nor has *EIN3* been observed in any yeast 1-hybrid by the PRESTA group (data not shown). It is possible *EIN3* is not a functioning protein in this variant of yeast 1-hybrid.

The protoplast reporter system was used to test the yeast 1-hybrid results. In some cases, results were successfully verified. The use of protoplasts enables more *in vivo* conditions, that can help to confirm and strengthen positive results observed in yeast 1-hybrid. This does not mean negative results in the protoplast transactivation reporter system imply false positive yeast 1-hybrid results - transcription factors may not have a native activating property, or may require different conditions to the ones present in protoplast cells. Therefore, positive results in yeast 1-hybrid are not invalidated by a negative protoplast-based result, but a positive protoplast assay can support yeast 1-hybrid data.

Negative yeast 1-hybrid results were not tested in the protoplast system, due to time and space constraints. However, it is possible that a number of transcription factors that did not bind to the *ANAC092* promoter fragments in yeast 1-hybrid

could have driven expression of the GUS reporter in the protoplast system had it been tested. The primary limit to using a protoplast system in a high-throughput manner is the generation of protoplast cultures and cost of extracting plasmids at the necessary purity (which requires maxiprep or midiprep kits). If these costs could be overcome then protoplast assays on a much larger scale could be conducted.

4.3.1. AP2/ERF Family Transcription Factors

A number of Ethylene Responsive Factor (ERF) family transcription factors bound to the 230bp region immediately upstream from the ATG of *ANAC092*. ERF family transcription factors are a subclade of APETALA2/ETHYLENE RESPONSE BINDING FACTORS (AP2/EREBP) transcription factors, distinguished by a single AP2 domain and a small number of introns (Nakano *et al.*, 2006). Despite their name, ethylene responsiveness is not a universal feature of these transcription factors, as some do not respond to ethylene and many respond to alternative stimuli such as dehydration or pathogen attack (Licausi *et al.*, 2013). Three ERF family transcription factors bound to the promoter of *ANAC092*; ORA59, ATERF14 and ESE1. These three transcription factors have previously been studied in multiple contexts.

ATERF14 is a known integrator of biotic stress signals. It is increased in expression in response to *Pseudomonas syringae* and ethylene treatment (Oñate-Sánchez & Singh, 2002), while mutants of *ATERF14* show increased susceptibility to *Fusarium oxysporum* (Oñate-Sánchez *et al.*, 2007). Expression of a number of defence related genes such as *PDF1.2*, *PR1* and *ChiB* are usually increased in response to ethylene treatment, however this function is abolished in *aterf14*, suggesting *ATERF14* regulates ethylene mediated biotic stress responses. ATERF14 may be responsible for transmitting biotic stress signals through ethylene signalling to *ANAC092* transcription.

ORA59 is a known integrator of ET/JA signals during pathogen attack, appearing to positively contribute to resistance to *Botrytis cinerea* by inducing defence related genes such as *PDF1.2* in an ET/JA dependent manner (Pré *et al.*, 2008; Zarei *et al.*, 2011). *ESE1* has not been shown to have a role in pathogen response. Instead, it appears to function in salt tolerance through an ethylene signalling pathway containing EIN2 and EIN3 (Zhang *et al.*, 2011). We can envisage a scenario where *ORA59* and/or *ATERF14* binds to the *ANAC092* promoter and drives expression of *ANAC092* during biotic stress, while *ESE1* induces expression of *ANAC092* during salt stress.

However, *ANAC092* was not identified as a differentially expressed gene 16 hours after induction of *ORA59* from an estradiole inducible promoter (Pré *et al.*, 2008). This tissue was not under stress conditions, therefore it is possible induction of *ANAC092* expression by ORA59 requires stress signals present during *Botrytis cinerea*

infection, such as ethylene or jasmonate signalling. Data from the protoplast transactivation assay described here suggests that ORA59 is capable of inducing a GUS reporter from an *ANAC092* promoter fragment, but at a low level. The protoplast system used in these experiments was similar to the inducible overexpressor experiment in Pré *et al.* (2008) in that it was not under stress conditions, aside from the treatment required to generate protoplast cells. The sudden increase in *ORA59* transcript levels was unable to induce *ANAC092* expression. However, it is possible ORA59 will be able to induce *ANAC092* transcription in the presence of other proteins or components not present here.

4.3.2. HD-Zip Family Transcription Factors

In the yeast 1-hybrid experiments *ATHB5* and *ATHB22*, two HD-Zip proteins, bound to the -153 to -317 region of the promoter. HD-Zip proteins belong to the Arabidopsis homeobox superfamily proteins, incorporating a homeodomain tightly linked to a leucine zipper, an arrangement unique to plants (Ruberti *et al.*, 1991; Schena & Davis, 1992). The Arabidopsis genome contains 47 HD-Zip genes, classified from 1 to 4 based on protein sequence and intron/exon organisation (Henriksson *et al.*, 2005). Like their animal counterparts, Arabidopsis homeobox genes are responsible for many developmental processes, but unlike their animal equivalent do not have a homeotic effect[1] (Ariel *et al.*, 2007). HD-Zip proteins hetero- or homodimerise via their leucine zipper domain prior to DNA binding (Sessa *et al.*, 1993; Meijer *et al.*, 2000; Johannesson *et al.*, 2001), suggesting they can mediate the activity of each other.

HD-Zip class 1 and 2 proteins have been shown to interact with a CAAT[A/T]ATTG pseudo-palindromic sequence (Sessa *et al.*, 1993; Meijer *et al.*, 2000; Johannesson *et al.*, 2001). Crucially, this sequence is located at 226-235bp upstream of the ATG of *ANAC092*. Arabidopsis HD-Zip 1 proteins have been shown to be strong transactivators in yeast (Meijer *et al.*, 2000) and in bombardment studies (Henriksson *et al.*, 2005), however neither *AtHB5* or *AtHB22* induced significant activation of the GUS reporter from the *ANAC092* promoter region (figure 4.11). The difference in results is possibly due to experimental design. In Henriksson *et al.* (2005), 6 repeats of the AtHB recognition site (CAATTATTG) were linked to a luciferase reporter. In the experiment conducted here, there is one repeat which is part of a larger 200bp promoter. The experiment described here is closer to an *in vivo* promoter region and may reflect native properties of an ATHB protein when associated with other transcription factors or proteins. It is possible AtHBs are strong activators when studied independently, but have alternative regulatory properties when part of the larger transcriptional unit.

[1] In animals, mutation or overexpression of homeotic genes leads to the transformation of one body part to another, known as the homeotic effect (Garber *et al.*, 1983).

Class 1 HD-Zip transcription factors are widely believed to be responsible for linking environmental stimuli to developmental events. Many are induced by external factors such as drought, osmotic stress, temperature and abscisic acid levels; in return they influence development such as embryogenesis or leaf polarity (Mayda *et al.*, 1999; Himmelbach *et al.*, 2002; Olsson *et al.*, 2004; Lin *et al.*, 2008; Manavella *et al.*, 2008). This possibly indicates that some HD-Zip proteins function as a link between stress and developmental signals, i.e., during stress they repress development in favour of stress response. ANAC092 can be considered to respond to developmental signals such as age-related senescence or lateral root formation and stress signals such as salt-stress or *Botrytis cinerea* infection. Therefore it seems plausible that the HD-Zip proteins ATHB22 and ATHB5 could regulate *ANAC092* expression during development or stress signals.

ATHB22 expression has not been detected in leaf tissue, but has been detected in seedlings and siliques of non-stressed plants (Henriksson *et al.*, 2005). *ATHB5* has been demonstrated to act as a positive regulator of ABA responsiveness during seedling establishment. ABA has a negative affect on seedling growth, restricting growth in unfavourable conditions (Lopez-Molina *et al.*, 2001). Overexpressors of *ATHB5* show reduced germination rate when ABA is applied, suggesting ATHB5 is a positive regulator of ABA signalling in the developing seedlings (Johannesson *et al.*, 2003). *anac092* mutants show an increased rate of seed germination under salt stress, while 35S:*ANAC092* lines show a greatly reduced rate of germination, suggesting *ANAC092* has the ability to suppress development under unfavourable conditions (Balazadeh *et al.*, 2010a). The similar phenotypes exhibited by *ANAC092* and *ATHB5* transgenic lines suggest they function in the same signalling pathway, while the yeast 1-hybrid data indicates there may be a direct connection between the two. Therefore, it is possible *ATHB5* acts as a link between environmental conditions and *ANAC092* expression during the early phase of the plants life. Overexpression of *ANAC092* or *ATHB5* reduced seed germination during stress conditions, suggesting ATHB5 and ANAC092 share a similar role in seeds. However, in our protoplast transactivation assay ATHB5 did not appear to induce expression of the GUS reporter from the *ANAC092* promoter fragment. On the contrary, it appeared to cause GUS expression to reduce, suggesting ATHB5 negatively regulates *ANAC092* expression. This was supported by the negative correlation of gene expression levels in the PRESTA timeseries data (figure 4.12).

The difference between the protoplast data and phenotypes could indicate that ATHB5 and ATHB22 can act as activators or repressors of expression, depending on other factors, such as binding proteins. Since *ATHB5* and *ATHB22* proteins have not been shown to have a role in leaves so far, it is possible that they repress transcription of target genes in the leaf mesophyll derived protoplast cells used in this experiment, hence the low expression levels observed in our protoplast assay. If the experiment was repeated using protoplasts generated from roots or seedlings it is possible we

may see different results. In the context of a developing Arabidopsis, this could indicate *ATHB5* influences *ANAC092* expression during seedling establishment, but has a reduced role in leaf tissue.

4.3.3. MYB Family Transcription Factors

MYB108 and *MYB116* bound to the *ANAC092* promoter in the yeast 1-hybrid system and drove expression of the GUS reporter from the ANAC092 promoter fragment in protoplasts. *MYB108* and *MYB116* are MYB proteins, the largest family of transcription factors in Arabidopsis (Stracke *et al.*, 2001; Dubos *et al.*, 2010). *MYB108* and *MYB116* share a high degree of similarity in their R2R3 MYB domains (71.6% by amino acid composition). *MYB108* is also known by the Arabidopsis mutant line *botrytis susceptible 1* (*bos1*), a T-DNA insert of *MYB108* characterised by hypersensitive Botrytis and wounding response (Mengiste *et al.*, 2003; Cui *et al.*, 2013). At a transcriptional level, the *MYB108* transcript is dramatically increased in *bos1*, but the phenotype of bos 1 plants can be restored to wild-type by heterozygosity, suggesting the transcript is non-functional (Mengiste *et al.*, 2003). The strong phenotype induced by alteration in *MYB108* transcript level indicates it is a major hub in response to *Botrytis cinerea* infection and wounding, however the role is unclear. Previously, *MYB108* have been identified to bind to the promoters of three other NAC transcription factors in yeast 1-hybrid and regulate them during *Botrytis cinerea* infection and dark-induced senescence (Hickman *et al.*, 2013), perhaps illustrating that *MYB108* targets a number of stress responsive NAC transcription factors, of which *ANAC092* may be included. *MYB116* was also observed to bind to the promoter of these NAC family transcription factors, but was not tested for a functional role. *MYB116* is expressed at negligible levels in all cells except roots (AtGenExpress data; Kilian *et al.*, 2007) which indicates that MYB116 does not have a role in the leaf. In Hickman *et al.* (2013), the closely related protein MYB2 was also found to bind to these promoters and have a alter transcription rates, however the same binding pattern was not seen with the *ANAC092* promoter, suggesting a subtle variation of the promoter region that is specifically recognised by MYB108 and MYB116.

4.3.4. NAC Family Transcription Factors

ANAC092 is one of the most studied members of the NAC transcription factors in *Arabidopsis*, but it is important to remember that it is a member of a large family with many uncharacterised members. Four NAC family transcription factors bound to the promoter of *ANAC092*; ANAC018, ANAC025, ANAC056 and ANAC102. *ANAC018*, *ANAC025* and *ANAC056* have not been studied in-depth before, but *ANAC102* has been identified as being expressed in response to oxygen stress. Mature *ANAC102* mutants have not been observed to have a particular phenotype

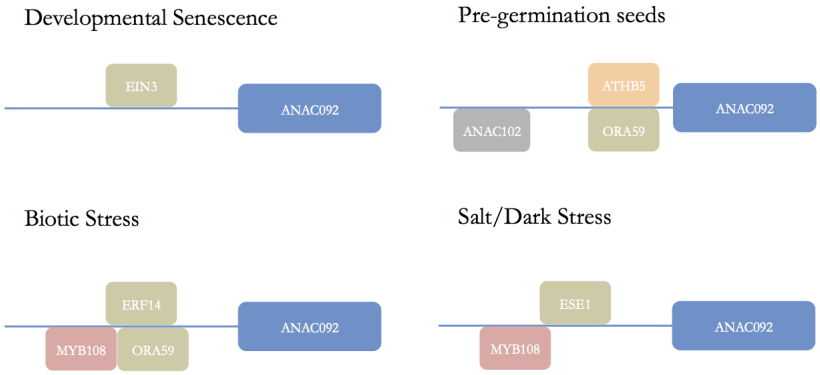


Figure 4.14: **Model for ANAC092 regulation from the promoter region** Possible model for regulation of *ANAC092* through the promoter region, determined by yeast 1-hybrid results and literature derived roles. Under differing conditions, different transcription factors are expressed or activated and bind to the promoter of *ANAC092*, causing it to be upregulated. A number of transcription factors identified in yeast 1-hybrid have not been assigned a function because they have not been studied previously.

(Christianson *et al.*, 2009), instead, *ANAC102* knock-out lines showed reduced germination rate in seedlings (Christianson *et al.*, 2009). *ANAC092* overexpressors show a reduced rate of germination under salt stress, so it is possible ANAC102 acts through *ANAC092* to repress germination and seedling establishment under stress conditions.

ANAC018, *ANAC025* and *ANAC056* are closely related, sharing roughly 80% amino acid sequence in their NAC domains, the region responsible for DNA binding. The highly conserved DNA binding domain probably recognises the same DNA motif on the *ANAC092* promoter. The protoplast assay showed all NAC proteins can induce expression of the GUS reporter from the *ANAC092* promoter fragment. Since the activation capability of NAC proteins appears to be dependent on their variable C-terminal domain, it is possible that proteins of similar DNA-binding properties have different transactivation capabilities. Furthermore, NAC proteins are known to form homo- and heterodimers. Therefore it is possible these NAC proteins are capable of binding to the *ANAC092* promoter in combination with each other or indeed other NAC proteins to modulate *ANAC092* expression.

4.3.5. Model of Regulation

In this chapter, a number of transcription factors have been identified that are capable of binding to the *ANAC092* promoter. Many of these transcription factors have been shown to have roles in regulating stress response where *ANAC092* expression increases. Therefore we can propose a potential model of regulation for multiple conditions in which *ANAC092* expression is promoted (figure 4.14). Under stress

conditions in seeds, *ANAC092* expression represses germination (Balazadeh *et al.*, 2010a). This may be regulated by ANAC102 promoting *ANAC092* expression. It is also possible ATHB5 and ATHB22 regulates expression of *ANAC092* in seeds, although it is not clear whether this is a positive or negative regulation. In premature senescence induced by salt or dark-treatment, *ANAC092* expression may be regulated by MYB108 and ESE1. Finally, during pathogenic infection, expression of *ANAC092* may be induced by *ATERF14*, *ORA59* and *MYB108*. This illustrates one of the strengths of using an *in vitro* system - since yeast 1-hybrid and protoplast assays are context free, they can identify protein:DNA interactions in the absence of specific *in vivo* conditions. The researcher is then able to analyse the role each protein:DNA interaction plays *in vivo*. The problem with this model is that it does not incorporate transcription factors that have not been studied previously, such as ANAC018 and ANAC025. The availability of complete transcriptome data allows us to infer novel functions for previously uncharacterised transcription factors. This has been successful earlier in this thesis to identify *ANAC092* as a gene involved in *Botrytis cinerea* response. Timeseries gene expression data can be exploited to predict how transcription factors binding to the promoter of *ANAC092* influence the expression levels. This is discussed in the next chapter.

5. Analysis of a transcriptional regulatory network for *ANAC092*

5.1. Introduction

The time course datasets generated by the PRESTA project are an invaluable tool for analysing stress responses in Arabidopsis. The high-resolution data allows temporal patterns of gene expression to be analysed across multiple stresses at a whole genome level. Underlying mechanisms can be predicted by monitoring clusters of genes whose expression is activated or inactivated in sequence, i.e., by monitoring the 'waves' of genes activated or repressed over time. In turn, network inference can be used to predict regulatory interactions between clusters. For example, in Windram *et al.* (2012), network inference was used to determine a hierarchy of gene clusters and illustrate a cascade of signalling. However, this failed to identify particular transcription factors responsible for linking the expression of these clusters because each transcription factor was obscured by every other transcription factor within the cluster. Analysis of the individual transcription factors capable of influencing changes is critical to understanding transcriptional responses to stress.

Because the PRESTA timeseries data are derived from whole genome microarrays, data are available for the majority of genes in Arabidopsis. As such, the expression profile of each gene can be utilised for prediction of molecular interactions between regulatory elements, known as a gene regulatory network (GRN). In principle, the gene expression profile of a gene will be influenced by the gene expression profile of transcription factors that regulate it, therefore rates of change in gene expression of both genes can be used to reverse engineer the network. This area has expanded rapidly in recent years in conjunction with increasing capabilities and reducing costs of whole '-ome' technologies such as microarrays, RNA-Seq, ChIP-chip and proteomics (D'haeseleer *et al.*, 2000; Moreno-Risueno *et al.*, 2010; Penfold & Wild, 2011; Swetlana Friedel, 2012).

PRESTA used the timeseries data in conjunction with the modelling algorithm Variational Bayesian Space State Modelling (VBSSM) to infer stress response networks between the transcription factors of Arabidopsis (Beal *et al.*, 2005). VBSSM represents a type of dynamic Bayesian network (DBN) modelling algorithm, which are more suitable for biological data over many other network inference techniques because of their ability to compensate for noisy or missing data (Murphy & Mian,

1999). The PRESTA project used VBSSM on the high resolution time course data for each stress to generate a large overlapping network composed of multiple smaller networks, such as the one shown in figure 5.1.

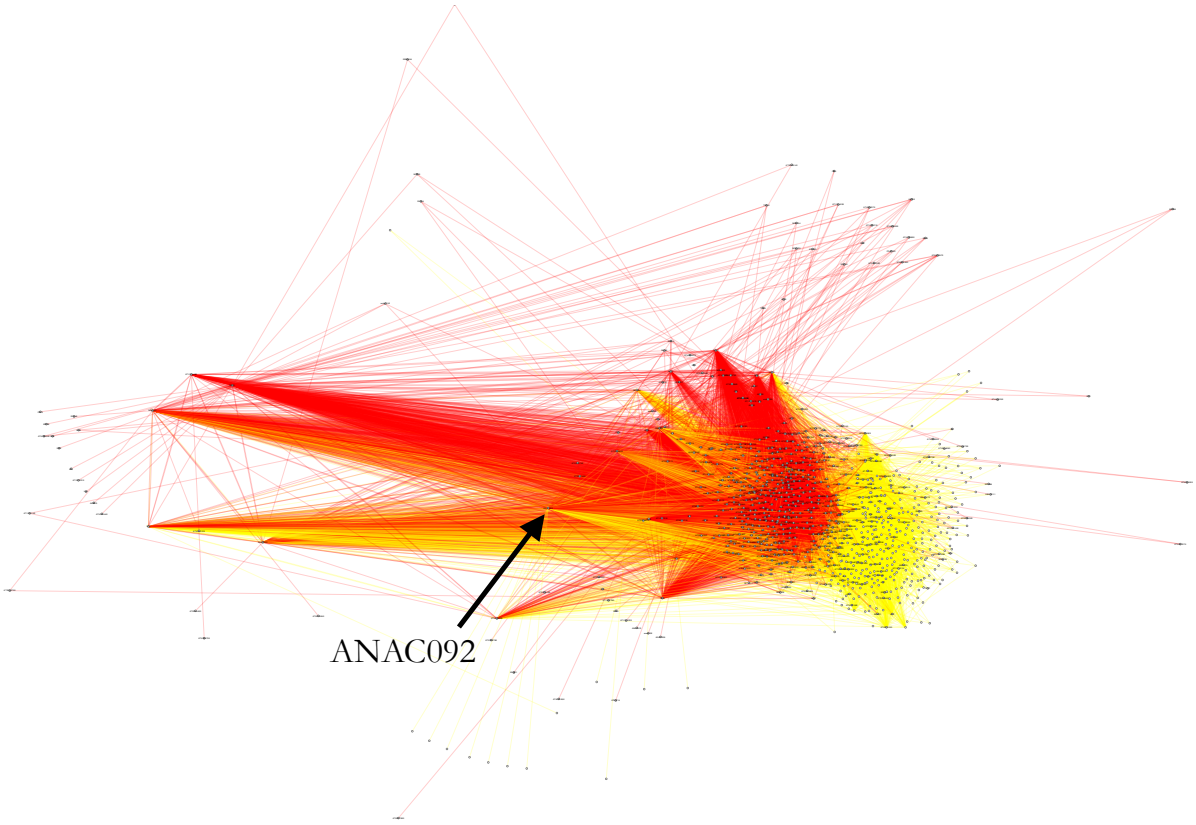


Figure 5.1: **MH-VBSSM model for Arabidopsis transcription factors during *Botrytis cinerea* infection and developmental senescence** Gene regulatory network for Arabidopsis stress responsive transcription factors generated by Metropolis-Hastings Variational Bayesian Space State Modelling (MH-VBSSM) using the *Botrytis cinerea* timeseries data and long-day senescence timeseries data from the PRESTA project. Yellow indicates an interaction in developmental senescence, while red indicates an interaction during Botrytis infection. *ANAC092* is labeled. The network has arranged using the 'organic' layout in Cytoscape 2.7, placing *ANAC092* as a central regulator between the two stress response signaling networks.

The networks generated by VBSSM illustrated a number of aspects about stress responsive transcription factors in Arabidopsis. Some of the transcription factors appear to be very specific for one stress, while others cross link multiple stresses. One of the key transcription factors that was identified to be involved in a number of stress responses was *ANAC092*, as it is a major hub in the networks derived from the *Botrytis cinerea* and senescence timeseries data. This is in agreement with the strong phenotype exhibited by *ANAC092* mutants and overexpressors during age-induced senescence and *Botrytis cinerea* infection (Oh *et al.*, 1997; Balazadeh *et al.*, 2010a, chapter 3 of this thesis), which suggests ANAC092 is critical for appropriate

response to these stresses.

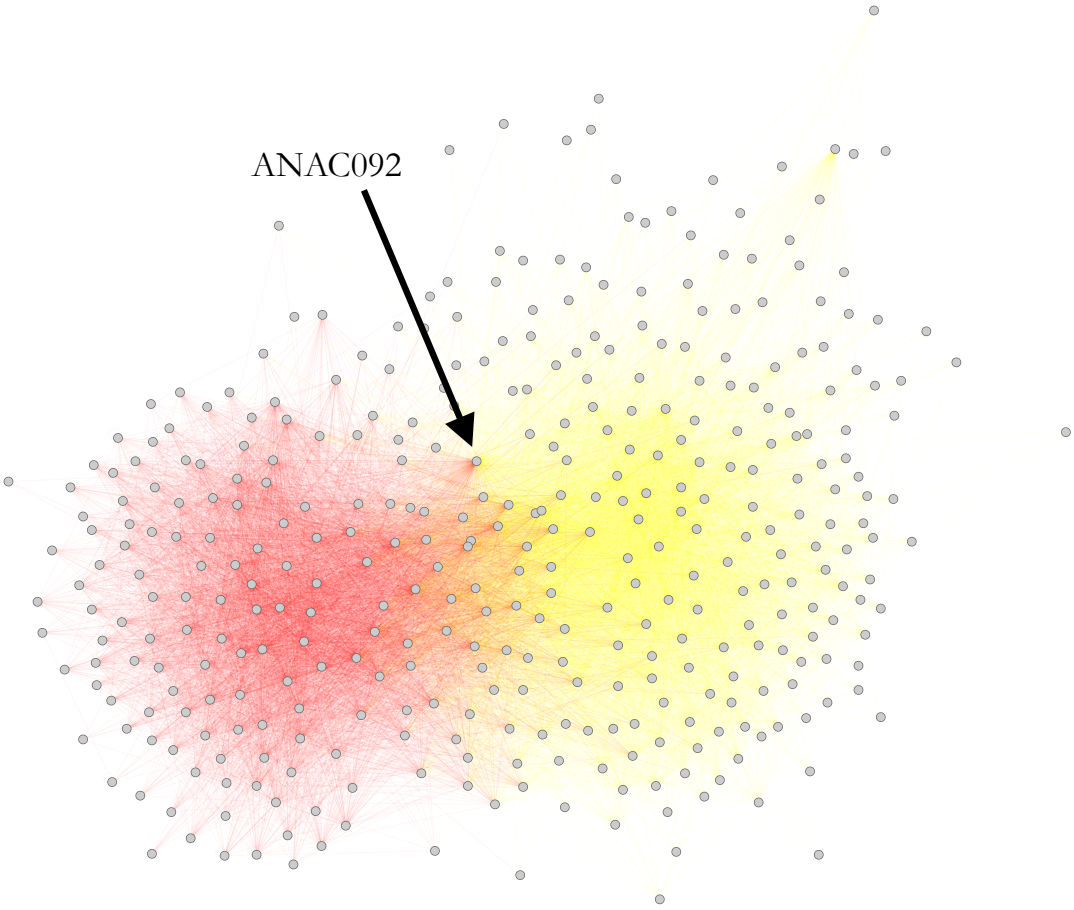


Figure 5.2: **First neighbors of ANAC092 in the MH-VBSSM model** First neighbors of *ANAC092* in MH-VBSSM from figure 5.1. Layout is 'organic' from Cytoscape 2.7, which has placed *ANAC092* as a central regulator between the two stress response signalling networks.

The natural extension from VBSSM modelling is to ask which genes in the network are predicted to regulate *ANAC092* expression and which genes are predicted to be regulated by ANAC092 activity. This is possible to visualise as the first neighbors of *ANAC092* (figure 5.2). This indicates a number of genes whose expression level may be directly linked to the expression level of *ANAC092*, which may refer to a biologically relevant regulation mechanism.

One problem with VBSSM and other network inference techniques is that they do not provide evidence for direct interactions between genes. For example, many genes are predicted to regulate *ANAC092* by the VBSSM model (figure 5.2), however it is not known which are directly regulating *ANAC092* transcription. This model needs to be supplemented with evidence of a physical interaction using complementary techniques.

In the previous chapter, a number of transcription factors were shown to bind to

the promoter of *ANAC092*, suggesting that they are capable of regulating *ANAC092* at a transcriptional level. Therefore, evidence for physical interactions between transcription factors and the *ANAC092* promoter has been identified. When comparing these data with the VBSSM model in figure 5.2, only a limited overlap is seen (figure 5.3). Therefore, although network inference by VBSSM is a powerful tool, it may struggle to identify predict direct regulation between two genes. Yeast 1-hybrid is effective at analysing protein:DNA interactions, but lacks *in vivo* conditions. Therefore it is impossible to know whether yeast 1-hybrid results are true for a particular context.

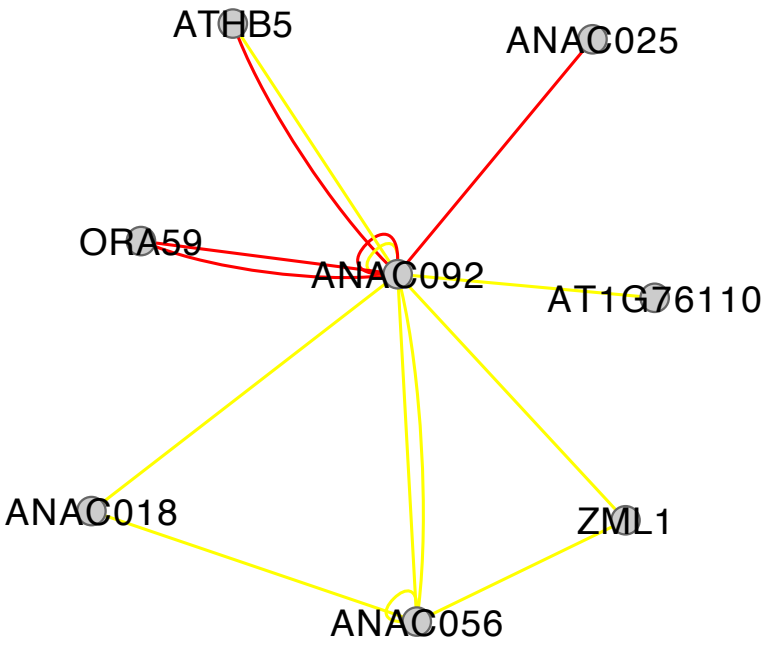


Figure 5.3: **Overlap between MH-VBSSM and yeast 1-hybrid data** Overlap between transcription factors identified as recognising the ANAC092 promoter region using yeast 1-hybrid results in the previous chapter and predicted first neighbors of *ANAC092* from MH-VBSSM in figure 5.2. Red lines indicate a prediction of interaction from *Botrytis cinerea* timeseries data, while yellow lines indicate a prediction from developmental senescence timeseries data.

The use of timeseries data and network inference in conjunction with yeast 1-hybrid may define *in vivo* contexts for *in vitro* yeast 1-hybrid connections and may be able to resolve small-scale gene regulatory networks, such as the one regulating *ANAC092* under multiple stress conditions.

5.1.1. Aim

The aim of this chapter was to identify and test potential roles for upstream regulators for *ANAC092*. PRESTA timeseries data and network inference was used to predict roles for transcription factors that were shown to bind to the promoter region of *ANAC092* in the previous chapter. These predictions were then tested with

transgenic Arabidopsis plants. Furthermore, a larger gene regulatory network was constructed using more yeast 1-hybrid data and network inference.

5.2. Results

5.2.1. PRESTA time series data predicts that regulators of ANAC092 have different roles during *Botrytis cinerea* infection and age-induced senescence

5.2.1.1. The transcription factors that regulate ANAC092 expression are differentially expressed in different stress treatments

In the previous chapter, 14 transcription factors capable of binding to the *ANAC092* promoter *in vitro* were identified. Some of these have been previously been studied and have a known function, however, others have not and therefore remain uncharacterised. For the transcription factors that have been studied, most have not been studied for a role in senescence or biotic stress and therefore their role in regulating *ANAC092* is difficult to predict. Since the expression profiles of every transcription factor that bound to the promoter of *ANAC092* is available in the PRESTA time-series data, predictions about their role during senescence and Botrytis infection can be made.

At the crudest level, if the mRNA of a transcription factor (or indeed, any gene) accumulates during a stress or condition, it is likely to be functional during that condition. This is because as the concentration of mRNA increases, it is likely the concentration of functional protein also increases and therefore there is functional protein present in the stressed tissue. Increased production of a particular gene in response to one condition is unlikely to be coincidental or meaningless and instead suggests an increase in concentration is beneficial for the plant to respond to the stress. Therefore identification of rapidly accumulating transcript or protein is often indicative of an important gene for the stress response. This methodology is frequently employed in research, with accumulation of a transcript often representing the first step to identifying the role of a particular gene.

The timeseries expression profile for the 14 transcription factors that were identified to bind to the promoter region of *ANAC092* are publicly available as part of Windram *et al.* (2012) for *Botrytis cinerea* infection and Breeze *et al.* (2011) for age-induced senescence. Many of these transcription factors are differentially expressed during Botrytis infection and/or age-induced senescence. An increase in the expression of a regulator may directly correlate with an increase in levels of the corresponding protein, therefore a gene that is differentially expressed during one condition may regulate *ANAC092* expression during that condition.

The transcription factors capable of binding to the *ANAC092* promoter in yeast 1-hybrid are shown in figure 5.4, with additional illustration showing whether they were identified as differentially expressed in *Botrytis cinerea* infection, age-induced senescence, both or neither (figure 5.4). *ESE1*, *ZML1* and *ANAC025* were differentially expressed during *Botrytis cinerea* infection, therefore they may be responsible

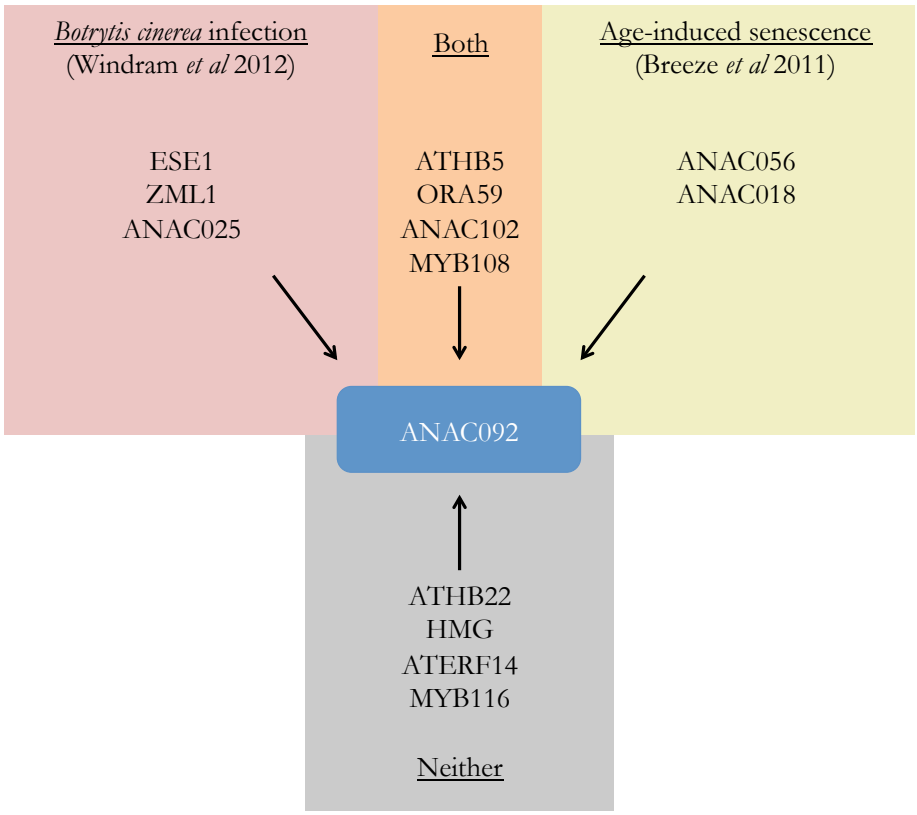


Figure 5.4: **Differential expression of potential regulators of *ANAC092* during *Botrytis cinerea* infection and age-induced senescence** Potential regulators of *ANAC092* as identified by yeast 1-hybrid, showing those that were identified as differentially expressed. The red box indicates genes that were identified as being differentially expressed during infection by *Botrytis cinerea* (Windram *et al.*, 2012), while the yellow box indicates genes identified as being differentially expressed during age-induced senescence (Breeze *et al.*, 2011). The orange box indicates a gene that was determined to be differentially expressed in both conditions, while the grey box indicates a gene that was not differentially expressed in either developmental senescence or *Botrytis cinerea* infection.

for causing *ANAC092* transcript to change during *Botrytis cinerea* infection. The two NAC transcription factors *ANAC018* and *ANAC056* were specifically differentially expressed during age-induced senescence, but not *Botrytis cinerea* infection, suggesting their transcript significantly changes over the time course of age-induced senescence and therefore they are potential regulators of *ANAC092* as the leaf ages. *ATHB5*, *ORA59*, *ANAC102* and *MYB108* were differentially expressed during both *Botrytis cinerea* infection and age-induced senescence, which means their expression dramatically changes during both conditions. It is possible these transcription factors regulate *ANAC092* expression during both stress conditions. Finally, *ATHB22*, *HMG (AT3G33550)*, *ATERF14* and *MYB116* were not determined to be differentially expressed during Botrytis infection or age-induced senescence, indicating their

transcript levels do not change over the course of these stress conditions. This may mean they are non-functional in these stresses, or alternatively they are regulated at a non-transcriptional level.

5.2.1.2. Co-expression of *ANAC092* and putative regulators

Differential expression indicates the transcript of a gene accumulates in a particular stress condition, which in turn can indicate the corresponding protein is active and functional in that tissue. However, it does not necessarily suggest that the gene binds to the promoter region of *ANAC092*. Many genes are expressed during *Botrytis cinerea* infection and senescence, but many of these are expressed after *ANAC092* transcript dramatically accumulates. If a gene is increased in transcription after *ANAC092*, it must have a reduced role in driving *ANAC092* transcription since the protein is not present at the appropriate time. As such, it is important to take into account the temporal patterns of expression for each of the transcription factors that may regulate *ANAC092*. The ideal profile of a regulator of *ANAC092* has a parallel expression profile, but with a slight lead time. This is because as the expression of the regulator increases, so do the corresponding target genes, allowing a small period of time for translation and activity.

The gene expression profiles of *ANAC092* and the putative regulators were compared (figure 5.5). Since it is important to see the change in gene expression, rather then the raw quantity, the gene expression profiles were used as log fold change with a mean of zero, to determine changes in expression levels, rather then absolute values. In addition, only *Botrytis cinerea* treated samples were used from the Botrytis dataset, since mock treated samples were not deemed to be relevant.

The gene expression profiles revealed some interesting facets of regulation. From the *Botrytis cinerea* data a number of genes clustered with *ANAC092*, suggesting their gene expression exhibited a similar pattern to *ANAC092* during the infection. This may indicate these genes regulate *ANAC092*. *ESE1*, *ORA59*, *ANAC025*E, *MYB108* and *ANAC102* begin to increase in expression levels at approximately 20 hours after infection, in a similar manner to *ANAC092*. Since their expression profiles reflect that of *ANAC092,* it may suggest there is a link between the two, therefore, they may regulate *ANAC092* during Botrytis infection.

ATHB5, *ZML1*, *ATHB22*, *MYB116* and *AT2G33550* cluster with a group of genes that show downregulation during *Botrytis cinerea* infection, suggesting these genes are 'switched off' over the timeseries. This could indicate one of two things. Since their expression decreases over the course of the infection, they may not contribute to transcription of *ANAC092* during Botrytis infection. Alternatively, they are negative regulators and repress transcription of *ANAC092.* During the infection, their levels decrease which relieves the repression of *ANAC092* transcription and allows *ANAC092* expression to increase. The protoplast transactivation system used in the

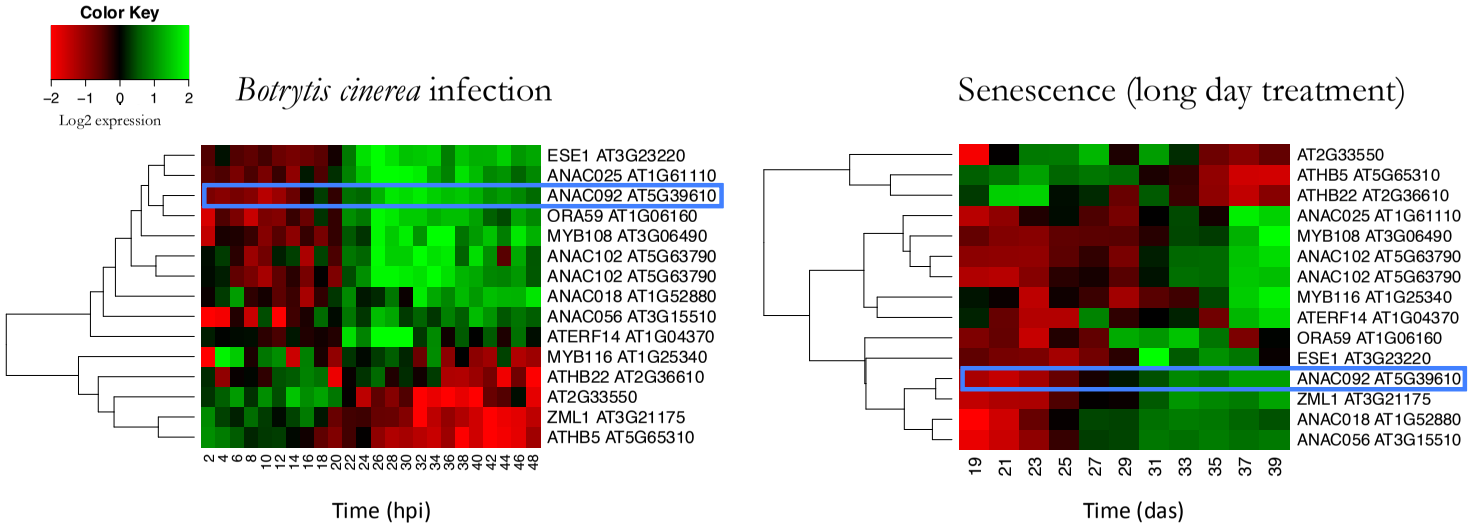


Figure 5.5: **Heatmap of *ANAC092* and potential regulator expression during *Botrytis cinerea* infection and senescence** Expression profiles of transcription factors that interact with the *ANAC092* promoter in yeast 1-hybrid and *ANAC092* (highlighted in blue). Red indicates downregulation while green indicates upregulation. hpi - hours post infection, das - days after sowing. Clustering performed in R using Euclidean distance matrix and complete agglomeration.

previous chapter indicated ATHB5 is a negative regulator of *ANAC092*. During a *Botrytis cinerea* infection, *ATHB5* transcript decreases which lifts the repression on *ANAC092* transcription and allows its expression to increase.

In the senescence dataset, similar patterns of clustering are observed, but different transcription factors cluster together. *ANAC018*, *ANAC056* and *ZML1* cluster with *ANAC092*, suggesting they are positive regulators of *ANAC092* expression, while *AT2G33550*, *ATHB22* and *ATHB5* expression levels decrease and therefore may be negative regulators of *ANAC092.* The transcription factors *ANAC025, MYB108, ANAC102, MYB116* and *ATERF14* all show minimal change in expression levels across the timeseries, although it does appear they increase in expression briefly before the end. This is not likely to indicate regulation of *ANAC092*, since their expression increases later then the increase in *ANAC092* expression.

The highly related transcription factors *ANAC018* and *ANAC056* show similar expression profiles during both age-induced senescence and *Botrytis cinerea* infection. *ANAC018* and *ANAC056* are the closest identified relatives of each other, while *ANAC025* is the next closest (Ooka *et al.*, 2003; Jensen *et al.*, 2010). This suggests these two transcription factors may be functional homologues of each other. *ANAC025* shows a highly divergent gene expression profile from the other two close NAC family members, indicating it may have diverged in function as well as protein sequence.

5.2.1.3. hCSI can be used to determine the role of known regulatory interactions

Analysis of changes in gene expression by-eye is a qualitative method of identifying possible regulatory genes. This means it is largely subjective and lacks robustness that can be determined using statistical methodologies. The use of network inference offers a more rigorous basis for determining the probability of a gene affecting expression of *ANAC092*.

The hierarchical Causal Structure Identification algorithm (hCSI, Klemm, 2008; Äijö & Lähdesmäki, 2009; Penfold *et al.*, 2012) is a network inference algorithm developed to test gene regulatory networks that have available timeseries data. It uses a prior network as a frame and uses timeseries datasets to predict whether edges within that network are more or less applicable in a particular stress. It has been previously used to predict gene regulatory networks for clusters of genes (Windram *et al.*, 2012) and for small yeast 1-hybrid networks (Hickman *et al.*, 2013) using timeseries data. Therefore it was decided to use this to determine the most likely interactions between *ANAC092* and its potential regulating transcription factors.

CSI is a form of network inference that uses non-parametric regression to infer gene regulatory networks. This means a Gaussian process is used to fit a nonparametric regression to the timeseries data of the regulator, which is then used to predict the

nonparametric regression of the target gene, also determined by a Gaussian process (Äijö & Lähdesmäki, 2009). The prediction can then be tested using Bayesian statistics. Non-parametric regression based techniques offer a number of advantages over ODEs or dynamic Bayesian networks as they do not require parameterisation or drastic assumptions to be made, but as a consequence require considerable amounts of data (Äijö & Lähdesmäki, 2009). As such it is appropriate to use when a timeseries profile is available for every gene in the network, with multiple replicates to counter for noise. The development by Penfold *et al.* (2012) to hierarchical CSI allows analysis of several distinct but overlapping networks, such as the one for Botrytis and senescence generated in this work.

The hCSI algorithm was applied to *ANAC092* and the transcription factors that were identified as capable of binding to the *ANAC092* promoter in yeast 1-hybrid, using PRESTA timeseries data for *Botrytis cinerea* infection and age-induced senescence. hCSI was used to predict interactions within the constraining yeast 1-hybrid network. There are separate marginal probabilities for *Botrytis cinerea* infection and age-induced senescence across all parent genes, which means it is possible to predict whether a transcription factor regulates *ANAC092* expression during *Botrytis* response and/or senescence.

5.2.1.4. hCSI predicts that different transcription factors regulate *ANAC092* during *Botrytis cinerea* infection and age-induced senescence

Figure 5.6A shows the marginal probabilities for each of the putative regulators of *ANAC092*. A marginal probability is on a scale from 0 to 1, with 1 being a complete positive result. A marginal probability of 1 would indicate the algorithm unanimously predicted the transcription factor regulated *ANAC092* expression, while a marginal probability of 0 would indicate the algorithm did not predict the transcription factor regulated *ANAC092*. The highest marginal probability was 0.26 (26%), suggesting that the algorithm frequently rejected a prediction that the transcription factor regulated *ANAC092* expression. This suggests that the hCSI algorithm was not able to suggest a certain transcription factor was capable of inducing *ANAC092* expression. This could indicate that none of the transcription factors highlighted here are regulators of *ANAC092*, or alternatively that hCSI was ineffective for predicting regulators of *ANAC092* expression.

The low hCSI predictions may mean it is of little value in predicting the ANAC092 regulatory network. However, it is important to note that the algorithm corroborated with the clustering performed previously (figure 5.5). In particular, *ESE1* has the highest marginal probability during Botrytis infection, suggesting it is a key regulator of *ANAC092* expression during this stress. This prediction is shared with the clustering analysis, which showed the expression of *ESE1* and *ANAC092* are correlated over time during a Botrytis infection. Similarly, the expression of *ANAC056*

(A)

ATG	Name	Age-induced Senescence	Botrytis
AT1G52880	ANAC018	0.07	0.01
AT1G61110	ANAC025	0.03	0.02
AT3G15510	ANAC056	0.24	0.01
AT5G63790	ANAC102	0.04	0.01
AT1G04370	ATERF14	0.06	0.01
AT2G36610	ATHB22	0.03	0.01
AT5G65310	ATHB5	0.09	0.01
AT3G23220	ESE1	0.03	0.26
AT1G76110	HMG	0.03	0.06
AT2G33550	Myb-hmg	0.18	0.01
AT3G06490	MYB108	0.04	0.01
AT1G25340	MYB116	0.08	0.01
AT1G06160	ORA59	0.04	0.08
AT3G21175	ZML1	0.03	0.14

(B)

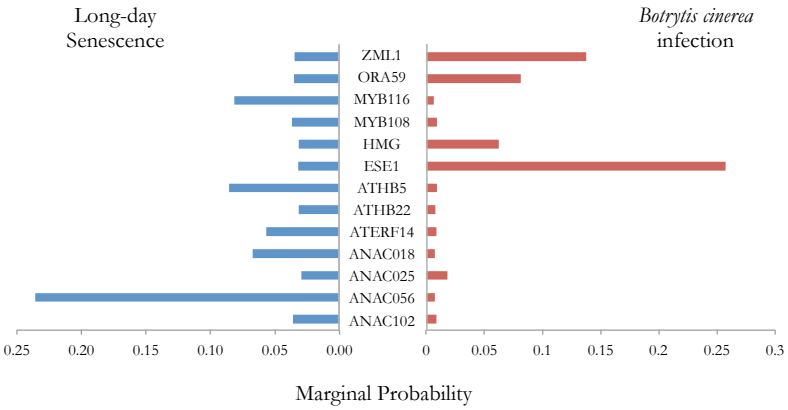


Figure 5.6: **Results of hCSI network inference** Numbers are marginal probabilities (i.e. the number of times the algorithm predicted each regulator would control *ANAC092* expression. (A) Raw marginal probabilities for each transcription factor. (B) Marginal probabilities for each regulator of *ANAC092*.

is correlated with *ANAC092* during age-induced senescence, while hCSI predicts *ANAC056* has the highest marginal probability during age-induced senescence.

5.2.1.5. Comparison of methods and predictions

The results obtained following hCSI (figure 5.6), clustering (figure 5.5) and gene expression comparison (figure 5.4) are largely in agreement with each other. ESE1 was predicted to be a regulator of *ANAC092* during *Botrytis cinerea* infection consistently, as was ANAC025 and MYB108. On the other hand, ORA59 looks plausible to be a regulator of *ANAC092* expression during *Botrytis cinerea* infection from the timeseries data, however hCSI did not predict this. It is known that ORA59 is a key regulator of response to *Botrytis cinere*a, promoting expression of *PDF1.2* and other stress response genes (Pré *et al.*, 2008; Zarei *et al.*, 2011), therefore it is likely that it is a key regulator of necrotrophic stress response. In the protoplast transactivation system in the previous chapter, constitutive expression of *ORA59* induced a small (although not significant) increase in *GUS* expression from the *ANAC092* promoter. Taken together, this may that imply although ORA59 is capable of binding to the

ANAC092 promoter, it does not 'drive' expression at a significant level, in *Botrytis cinerea* infection or otherwise.

For age-induced senescence, ANAC056 was consistently predicted to be a regulator of *ANAC092* expression. This implies that ANAC056 is a major regulator of *ANAC092* during age-induced senescence. The close homologue ANAC018 was predicted to be a regulator of *ANAC092* during age-induced senescence by both differential gene expression and comparison of gene expression profiles. *ANAC018* is a close relative of *ANAC056*, with a very similar expression profile and therefore it might be expected to have a redundant or highly overlapping function. As such, it may have a similar role in controlling *ANAC092* expression during Botrytis infection.

The MYB family transcription factor MYB108, is a well known regulator of responses to *Botrytis cinerea* amongst other necrotrophic pathogens (Mengiste *et al.*, 2003; Kraepiel *et al.*, 2011; Cui *et al.*, 2013). It is differentially expressed during *Botrytis cinerea* infection and the expression profile of *MYB108* is in agreement with it being a regulator of *ANAC092*, i.e., the expression increases shortly before *ANAC092* expression. The phenotype of a *bos1* during *Botrytis cinerea* infection is similar to the phenotype of plants overexpressing *ANAC092* (chapter 3 of this thesis), which enhanced cell death and enlarged lesions. The similar phenotype exhibited by the two lines may indicate they act in the same pathway. During senescence, expression of *MYB108* increases concurrent with *ANAC092*, suggesting it may be a positive regulator of *ANAC092* expression.

MYB116 is a close relative of *MYB108*, but has not previously been studied in depth. *MYB116* expression does not change during Botrytis infection or age-induced senescence, which might mean that *MYB116* is not a dynamic regulator of *ANAC092*. There are no available T-DNA insertion lines for *MYB116* from SALK (Alonso *et al.*, 2003), SAIL (Sessions *et al.*, 2002) or WiscDsLox (Woody *et al.*, 2006) libraries and no transgenic plants of *MYB116* have been published, possibly indicating that correct MYB116 function may be necessary for plant viability.

Interestingly, the transcription factor ZML1 was predicted to be a key regulator of *ANAC092* during age-induced senescence and *Botrytis cinerea* infection, in a positive and negative manner respectively. During age-induced senescence, *ZML1* expression was positively correlated with *ANAC092* expression, suggesting it is a positive regulator of *ANAC092*. However, during *Botrytis cinerea* infection, *ZML1* expression is negatively correlated with *ANAC092* expression, suggesting it is a negative regulator of *ANAC092*. It is possible that ZML1 acts as both a transcriptional activator and repressor, depending on the activity of other intracellular components.

5.2.1.6. Different protein combinations may interact with the *ANAC092* promoter during *Botrytis cinerea* infection and age-induced senescence

In the previous chapter, it was suggested that a single binding motif might function in different stresses by recruiting different members of the same transcription factor family. One example was that ATERF14 and/or ORA59 may be responsible for binding to the promoter of *ANAC092* during *Botrytis* infection, while ESE1 may bind during salt-induced senescence.

With the analysis of timeseries data, this idea can be extended to *Botrytis cinerea* infection and age-induced senescence. Figure 5.7 summarises the predictions that each binding transcription factor may regulate *ANAC092* expression during *Botrytis cinerea* infection or age-induced senescence.

ORA59 and ATERF14 are predicted to regulate *ANAC092* expression during *Botrytis cinerea* infection, as suggested by the literature (Oñate-Sánchez *et al.*, 2007; Pré *et al.*, 2008). Previous work has identified ESE1 as functioning in salt stress, but gave no indication to its role during *Botrytis cinerea* infection or developmental senescence. The timeseries data suggests that ESE1 may function in *Botrytis* infection and not developmental senescence. *ESE1*, *ATERF14* and *ORA59* are responsive to ethylene (Oñate-Sánchez *et al.*, 2007; Pré *et al.*, 2008; Zhang *et al.*, 2011), as is *ANAC092* (Kim *et al.*, 2009, 2011, 2014). Ethylene is a known component of biotic stress response (Thomma *et al.*, 1999; Lorenzo *et al.*, 2003) and this result might suggest an ethylene based signalling cascade to *ANAC092* expression through the action of ERF proteins during *Botrytis cinerea* infection.

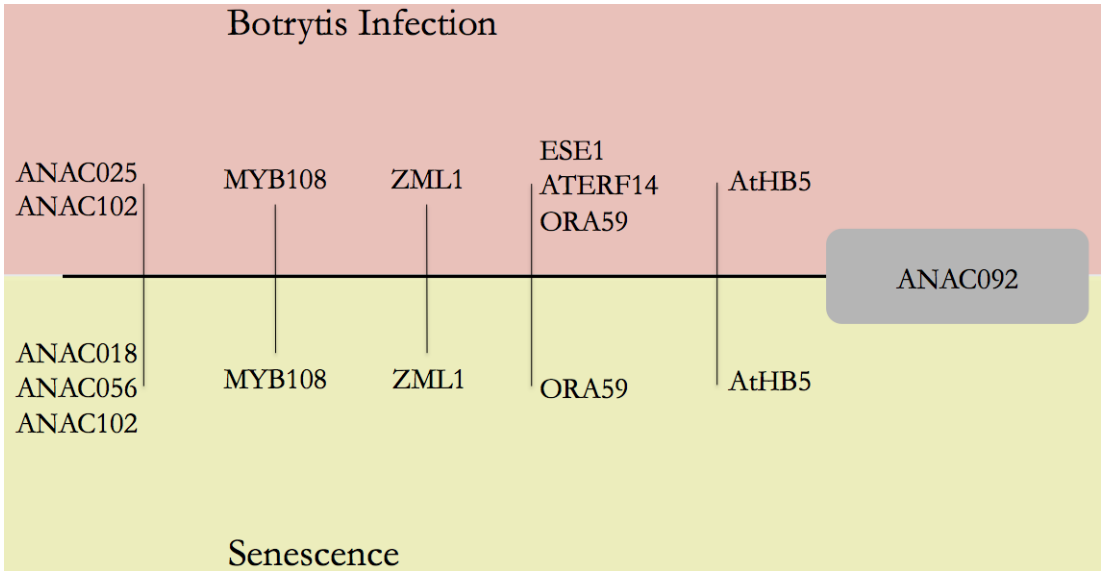


Figure 5.7: **Predictions of transcription factor regulation on *ANAC092* expression** Yeast 1-hybrid results for the upstream genomic region of *ANAC092,* showing those that are predicted to regulate *ANAC092* during *Botrytis cinerea* infection (red box) or age-induced senescence (yellow box).

MYB108 and ATHB5 are predicted to regulate *ANAC092* during both *Botrytis cinerea* infection and age-induced senescence, but their close relatives MYB116 and ATHB22 are not predicted to regulate *ANAC092* in either condition. This may suggest that while the other transcription factors may have a role in regulating *ANAC092*, it is not in the context of senescing leaf tissue or during pathogenic stress.

For the NAC family transcription factors, a distinct clade of binding proteins are seen to bind to the promoter of *ANAC092*. These proteins are predicted to deviate in function. ANAC025 is predicted to regulate *ANAC092* specifically during *Botrytis cinerea* infection, while ANAC018 and ANAC056 are predicted to regulate *ANAC092* specifically during age-induced senescence. ANAC102 is predicted to be functional during both stress conditions. NAC proteins are known to form homo- or heterodimers, therefore it is possible that different combinations of these transcription factors bind to the promoter region of *ANAC092* during different stress conditions.

Since one group of genes is suggested to bind to the upstream genomic region of *ANAC092* and regulate expression during *Botrytis cinerea* infection, while a separate group appears to have this role during senescence, it is possible that *ANAC092* expression requires a combination of these proteins. It is also possible that these transcription factors associate to form a large complex which promotes the formation of the basal transcription unit. The formation of transcription factor protein complexes on DNA is poorly understood in plants, although a few examples have been identified (Fan & Dong, 2002; Després *et al.*, 2003; Tran *et al.*, 2007).

Yeast 1-hybrid, gene expression analysis and hCSI modelling have been used to predict a number of transcription factors to play a role in determining the gene expression levels of *ANAC092* during age-induced senescence and/or *Botrytis cinerea* infection. Some of these predictions were then tested using a variety of methods.

5.2.2. Expression of *ANAC092* is reduced in *myb108-1*

MYB108 is a stress responsive gene, which has been shown to have a role in both response to *Botrytis cinerea* and mechanical wounding (Mengiste *et al.*, 2003; Cui *et al.*, 2013). In addition, it has a role in jasmonate mediated stamen maturation triggered through *MYB21*, shown by reduced fertility in *MYB108* mutants (Mandaokar & Browse, 2009). In the PRESTA timeseries data *MYB108* is differentially expressed during *Botrytis cinerea* infection and long day senescence. MYB108 binds to the *ANAC092* promoter in yeast 1-hybrid and has a similar expression profile during *Botrytis cinerea* infection and developmental senescence, suggesting a correlation between the expression levels of the two genes (figure 5.8). This may be caused by these two genes being coregulated, or alternatively it may be caused by one gene influencing the expression of the other directly.

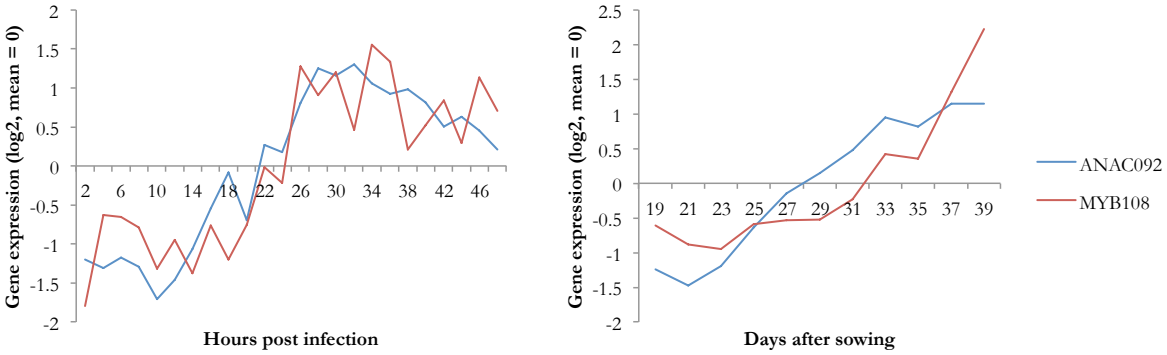


Figure 5.8: **Comparison of *MYB108* and *ANAC092* expression levels during *Botrytis cinerea* infection and age-induced senescence** PRESTA time-series data for *MYB108* and *ANAC092* over long day (left) and during *Botrytis cinerea* infection (right), plotted on the same graph.

MYB108 has previously been shown to bind to the promoters of three other NAC family proteins (Hickman *et al.*, 2013). These three transcription factors were reduced in expression levels during *Botrytis cinerea* infection and during dark-induced senescence in a *myb108* knock-out line (*myb108-1,* SALK-076395). The suggestion is that absence of MYB108 causes expression levels of the MYB108 targets to diminish, due to the lack of transcription factor promoting their expression. Gene expression levels in the *MYB108* mutant were determined using CATMA microarrays and so the gene expression levels of *ANAC092* can be examined from the same dataset. The gene expression data from the *MYB108* knock-out during *Botrytis cinerea* infection and dark-induced senescence were analysed and *ANAC092* expression levels were compared in *myb108-1* relative to Col 0. There were three time points for *Botrytis cinerea* infection (24, 28 and 32 hours post infection) and 2 time points for dark-induced senescence (8 & 9 days post treatment). *ANAC092* transcript levels were reduced at all time points during *Botrytis cinerea* infection although not significantly based on a linear model fit (Wettenhall & Smyth, 2004). During dark-induced senescence, *ANAC092* transcript was significantly reduced at both time points during dark induced senescence (table 5.1).

The results indicate that in *MYB108* knock-out line, the absence of *MYB108* has caused a downregulation of *ANAC092* transcript in both dark-induced senescence and *Botrytis cinerea* infection. This suggests that *MYB108* is required for normal *ANAC092* expression during dark-induced senescence.

It is important to note that although the change in *ANAC092* expression levels may have been caused by the *myb108-1* knock out, they might also have been caused by a phenotype that *myb108-1* caused, i.e., if a *myb108-1* knockout line has a delayed senescence phenotype, expression of senescence related genes (including *ANAC092*) may be induced later. *myb108-1* was analysed for a phenotype in *Botrytis cinerea*

	Time	Log2 Fold Change	P.Value	Adj.P.Val
Botrytis cinerea infection	26 hpi	-0.25	1.43E-01	6.96E-01
Botrytis cinerea infection	30 hpi	-0.42	1.04E-02	1.50E-01
Botrytis cinerea infection	34 hpi	-0.44	1.31E-02	2.33E-01
Dark treatment	5 dpt	-1.20	8.78E-05	5.16E-03
Dark treatment	6 dpt	-1.99	7.75E-06	1.72E-04

Table 5.1: **Expression of *ANAC092* in *myb108-1* during *Botrytis cinerea* infection and dark-induced senescence** Expression levels of *ANAC092* in *MYB108* knock-out line compared to Col 0 as determined by microarrays in Hickman *et al.* (2013). Times are hours post infection (hpi) or days post treatment (dpt). p-values were determined by linear model fitting, which is corrected by Benjamini-Hochberg correction (Benjamini & Hochberg, 1995).

infection and during dark-induced senescence (figure 5.9). *myb108-1* showed a reduced lesion size during *Botrytis cinerea* infection, suggesting *MYB108* contributes to the *Botrytis* infection and the lack of MYB108 in *myb108-1* causes a resistant phenotype. During dark-induced senescence, *myb108-1* showed an accelerated rate of senescence, as determined by R:G ratio of leaf 5. This suggests *MYB108* inhibits senescence during dark-treatment.

myb108-1 exhibited a similar phenotype to *anac092-1* during *Botrytis cinerea* infection. Concurrently, *ANAC092* expression was reduced in *myb108-1* during Botrytis infection (table 5.1). This indicates that *ANAC092* transcript may be reduced in *myb108-1* during Botrytis infection due to the reduced spread of infection, rather than a direct effect.

During dark-induced senescence, *myb108-1* shows accelerated senescence while *ANAC092* mutants show delayed senescence (Oh *et al.*, 1997). Expression of *ANAC092* was reduced in *myb108-1* during dark-treatment (table 5.1). In wild type plants, the level of *ANAC092* expression is positively correlated with senescence, however in the *myb108-1* plants *ANAC092* expression levels have reduced, despite senescence being accelerated. Therefore, we can suggest that reduction in *ANAC092* expression in *myb108-1* during dark-treatment is due to the lack of functional *MYB108* transcript, rather then an indirect effect on phenotype.

5.2.3. Influence of four NAC family transcription factors on *ANAC092* expression

5.2.3.1. Four NAC transcription factors appear to have diverging roles in stress response when regulating expression of ANAC092

In the previous chapter, it was shown that four NAC transcription factors could be recruited to the *ANAC092* promoter in yeast 1-hybrid. It was also shown that these transcription factors were capable of inducing expression a *GUS* reporter from *ANAC092* promoter fragments in a protoplast transactivation system. However,

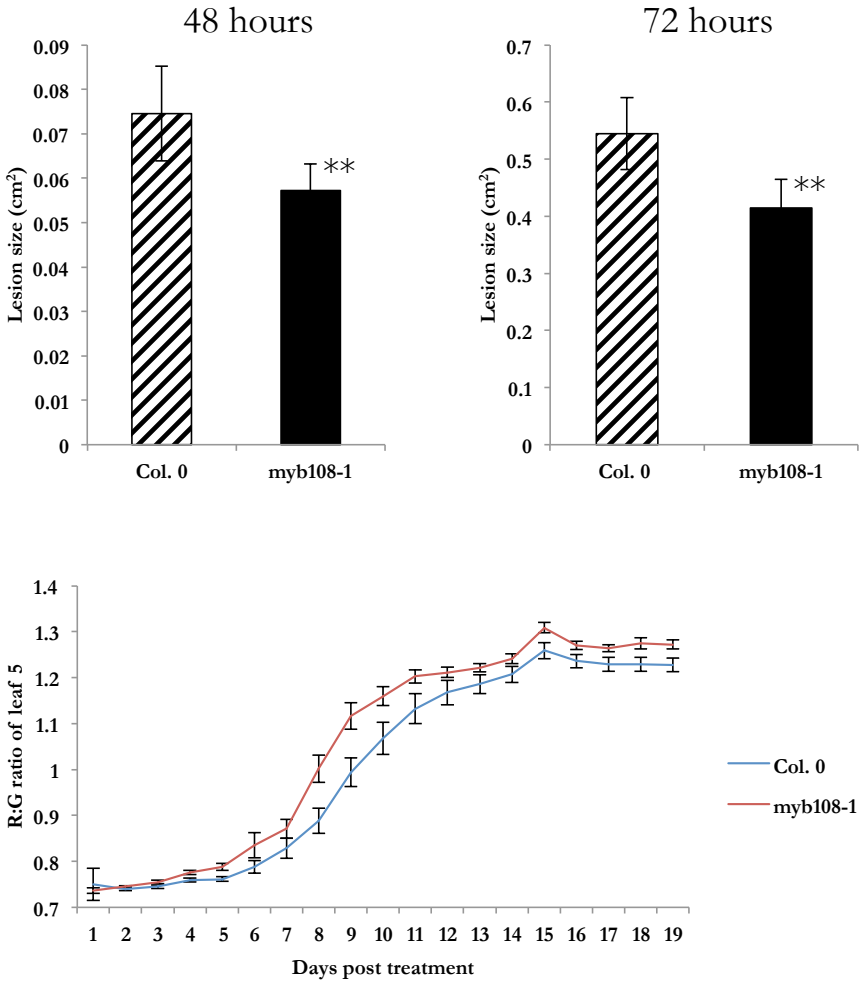


Figure 5.9: **Phenotypes of *myb108-1* during Botrytis infection and dark-induced senescence** (Top) Lesion size of *Botrytis cinerea* infection on Col 0 and *myb108-1*. Error bars represent SEM (N=30), ** indicates $P < 0.01$ on a t-test. (Bottom) R:G ratio of leaf 5 of Col. 0 and *myb108-1* during dark treatment. Error bars represent SEM (N=9)

modeling and gene expression analysis revealed that the NAC transcription factors appear to be split in function. Overall, the predictions seemed to indicate that ANAC056 and ANAC018 regulate *ANAC092* during developmental senescence, while ANAC025 regulates *ANAC092* during Botrytis infection. ANAC102 appears to regulate *ANAC092* during under both conditions. These predictions were tested using transgenic lines of these NAC proteins.

Name	Gene ATG	CATMA ID	$\log_2$ FC	p-Value	Adj. p-value
ANAC056	AT3G15510	CATMA3A14920	4.11	4.67E-05	0.047
ANAC092	AT5G39610	CATMA5A35200	0.17	0.45	0.82

Table 5.2: **Expression of *ANAC056* and *ANAC092* in an *ANAC056* overexpressor** Expression levels of *ANAC056* and *ANAC092* in 28 day old rosettes of Arabidopsis constitutively expressing *ANAC056*, as determined by CATMA V4 microarray (Sclep *et al.*, 2007). p-values determined by a linear model fit before a Benjamini and Hochberg correction (Benjamini & Hochberg, 1995).

5.2.3.2. Overexpression of *ANAC056* causes increased expression of a number of stress responsive genes

A simple molecular method to test whether one gene can influence the expression of another gene is to alter the natural expression levels of the predicted regulator. If a regulator of *ANAC092* is constitutively expressed in Arabidopsis, induced expression of *ANAC092* may be detected. A homozygous transgenic line expressing the *ANAC056* coding region from the CaMV35S constitutive promoter was kindly donated by Jesper Gronlund. The plant showed a significant dwarf phenotype and therefore was not suitable for phenotype analysis under a particular stress. However, 28 day old whole rosettes were taken for gene expression analysis. RNA was extracted from five plants of 35S:ANAC056 and Col 0 at 28 days old before gene expression changes were compared using a CATMA V4 microarray (Sclep *et al.*, 2007).

ANAC056 expression was dramatically increased in the overexpressor ($\log_2$ fold change = 4.1, roughly 17 times expression in Col 0, table 5.2). Expression of *ANAC092* was mildly upregulated in the *ANAC056* overexpressor ($\log_2$ fold change = 0.17, table 5.2), but not significantly (Adj. p-value 0.82). 48 genes were significantly upregulated while 23 genes were significantly downregulated. The *ANAC056* overexpressor had a dwarf phenotype, therefore it may be that gene expression differences were related to repression of growth signals and upregulation of stress signals. Enrichment of GO terms associated with the up- or downregulated genes was determined using the BiNGO plugin for cytoscape (Maere *et al.*, 2005). A number of stress related GO terms were enriched in the upregulated genes, such as response to water stress and glucosinolate metabolism (table 5.3). Meanwhile, a number of metabolism and photosynthetic GO terms such as thylakoid, chloroplast and photosynthesis related genes were downregulated in the *ANAC056* overexpressor (table 5.4). This suggests *ANAC056* promotes stress response and restricts photosynthesis, leading to the dwarf phenotype. The downregulation of genes relating to photosynthesis and chloroplast development is a key process in the onset of senescence, therefore it is possible ANAC056 is promoting aspects of senescence prematurely, leading to a retarded growth rate. However it is important to note that it is impossible to determine whether *ANAC056* is driving expression of these genes or whether overproduction of *ANAC056* is stressful for the plant, leading to downstream transcriptional responses.

ANAC092 expression was not significantly affected in the overexpressor of *ANAC056*. This could be due to a variety of factors. It is possible that ANAC056 may require cofactors or other transcription factors to induce expression of *ANAC092*. This has been seen previously with the NAC transcription factor ANAC019, which is not able to drive expression of *ERD1* on its own, but requires the addition of ZFHD1 (Tran *et al.*, 2007). If this is true, it would imply ANAC056 is capable of driving expression of genes other then *ANAC092* without the second protein, as shown by the changes in gene expression in the microarray. However it may require another protein to drive expression of ANAC092. This may be one of the other transcription factors shown to bind to the *ANAC092* promoter in yeast 1-hybrid previously.

GO-ID	Adj. p-Value	Description
9414	1.02E-03	Response to Water Deprivation
5576	1.08E-03	Extracellular Region
9607	1.08E-03	Response to Biotic Stimulus
16143	1.18E-03	S-Glycoside Metabolic Process
19760	1.18E-03	Glucosinolate Metabolic Process
42221	1.55E-03	Response to Chemical Stimulus
16137	2.69E-03	Glycoside Metabolic Process

Table 5.3: **GO terms overrepresented in the set of upregulated genes in an *ANAC056* overexpressor** Genes determine to be significantly upregulated in plants overexpressing *ANAC056* by CATMA V4 microarray were analysed for overrepresented GO terms using BiNGO (Maere *et al.*, 2005), adjusted p-values have been corrected using a Benjamini and Hochberg correction (Benjamini & Hochberg, 1995).

GO-ID	Adj. p-Value	Description
44434	7.26E-08	Chloroplast Part
9570	2.49E-05	Chloroplast Stroma
15979	3.10E-05	Photosynthesis
9534	3.10E-05	Chloroplast Thylakoid
9628	4.29E-05	Response to Abiotic Stimulus

Table 5.4: **GO terms overrepresented in the set of downregulated genes in the *ANAC056* overexpressor** Genes determine to be significantly downregulated in plants overexpressing *ANAC056* by CATMA V4 microarray were analysed for overrepresented GO terms using BiNGO (Maere *et al.*, 2005), p-values have been corrected using a Benjamini and Hochberg correction (Benjamini & Hochberg, 1995).

5.2.3.3. T-DNA insert lines of *ANAC018*, *ANAC025*, *ANAC056* and *ANAC102*

Constitutive expression of *ANAC056* conferred a strong phenotype, but gene expression analysis had not demonstrated a direct influence of *ANAC056* expression levels on *ANAC092*. It is likely overexpression of the NAC transcription factors will cause

developmental defects, making them inappropriate for studying regulation . Instead, it was decided to use knock-out lines showing reduced expression of the putative regulators of *ANAC092*. The primary advantage of a gene mutant is that if the gene is expressed at a minimal level in the plant, the lack of functional protein has a minimal effect. However, where the gene is expressed at high levels, the elimination of functional transcript or protein has a significant effect. In the context of stress responsive transcription factors, this means developmental defects in mutants are kept to a minimum, due to the minimal effect of the mutation in unstressed plants.

T-DNA insertion lines were obtained for the four NAC proteins, composed of SALK and WiscDsLox lines (figure 5.10, Alonso *et al.*, 2003; Woody *et al.*, 2006). Where possible, lines were identified where the T-DNA had inserted into an exon and therefore directly disrupted the coding sequence of the gene. This was not possible for *ANAC056*, which had a T-DNA insert in the untranslated 3' end (see figure 5.10), however, this line had been used previously by Claire Hill and Emily Breeze successfully, therefore it was included. The presence of correct homozygous inserts was confirmed by PCR from either side of the genomic region (data not shown).

Ideally, a T-DNA insert will prevent the formation of an active transcript and therefore abolish gene function, however, it is possible that truncated transcripts may retain some activity. Therefore PCR was used to determine levels of mRNA present. PCR of the transcript before visualisation on a gel is often used to quantify gene expression levels in mutant and wild-type plants. Since this does not produce a numerical value for each gene this is often referred to as semi-quantitative PCR. This technique has low sensitivity, and often struggles to quantify gene expression of low-abundance transcription factors. As such, quantitative PCR (qPCR) was used. The use of SYBR green allows qPCR to be far more sensitive then semi-quantitative PCR, therefore it can be used to compare the levels of low-abundance transcription factors. The quantification of gene expression levels of the four NAC mutants were determined using qPCR. The primer sequences and PCR program are given in the methods section.

Detection of a true knockout is dependent on the gene being expressed at detectable levels in the wild-type line. Many stress responsive transcription factors are expressed at low/negligible levels in unstressed tissue, therefore attempting to detect them would simply result in low expression for both the wild type and knock-out line. Therefore, genes were analysed for expression in tissue that should be producing substantial expression of the gene, determined by the PRESTA timeseries and publicly available data (figure 5.10B). Senescent leaves of 42 day old plants were analysed for *ANAC018* and *ANAC056* expression in *anac018-1*, *anac056-1* plants respectively. Expression of *ANAC018* was diminished by 73% compared to Col. 0, while expression in *ANAC056* was diminished by 53% compared to Col. 0. *ANAC025* expression was tested at 30 hours post infection with *Botrytis cinerea* infection. Expression of *ANAC025* was diminished by 90% in the *anac025-1* line. This suggests these T-DNA

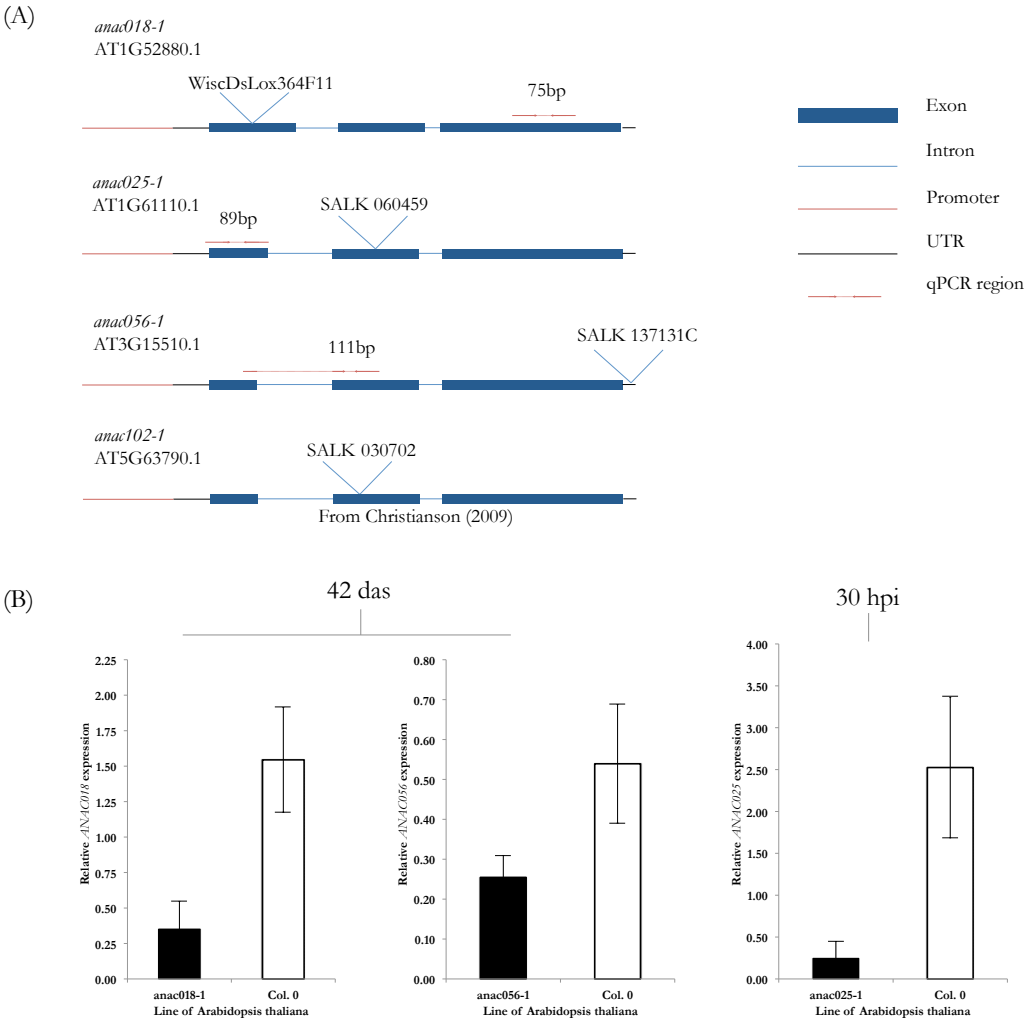


Figure 5.10: **T-DNA insertions for *ANAC018*, *ANAC025*, *ANAC056* and *ANAC102*** Description T-DNA insert lines of the four NAC genes. (A) The genomic region for each of the four NAC genes, showing introns, exons, location of the T-DNA insert and region amplified for qPCR genes. (B) Shows the relative expression of *ANAC018*, *ANAC056* and *ANAC025* in their respective knock-out lines, as determined by qPCR. *anac018-1* and *anac056-1* were tested in leaf 7 of 42 day old plants (senescent tissue), while *anac025-1* was tested in leaf 7 of *Botrytis cinerea* infected plants. *anac102-1* was not tested as it had previously been shown to be a true mutant in Christianson *et al.* (2009). Error bars represent SEM (N=3).

lines have reduced expression for their respective NAC protein.

5.2.3.4. *anac025-1* shows an altered phenotype during *Botrytis cinerea* infection

The predictions from the timeseries data were *ANAC025* and *ANAC102* promotes expression of *ANAC092* during *Botrytis cinerea* infection, but *ANAC018* and *ANAC056*

do not. It is possible that if a gene promotes *ANAC092* expression during *Botrytis cinerea* infection, elimination of that gene will cause a similar phenotype, i.e., it will induce a partial/full reduction of *ANAC092* transcription which simulates a similar phenotype to the *ANAC092* knock-out line. Therefore all four NAC gene knock-out lines were tested for phenotypic changes during *Botrytis cinerea* infection.

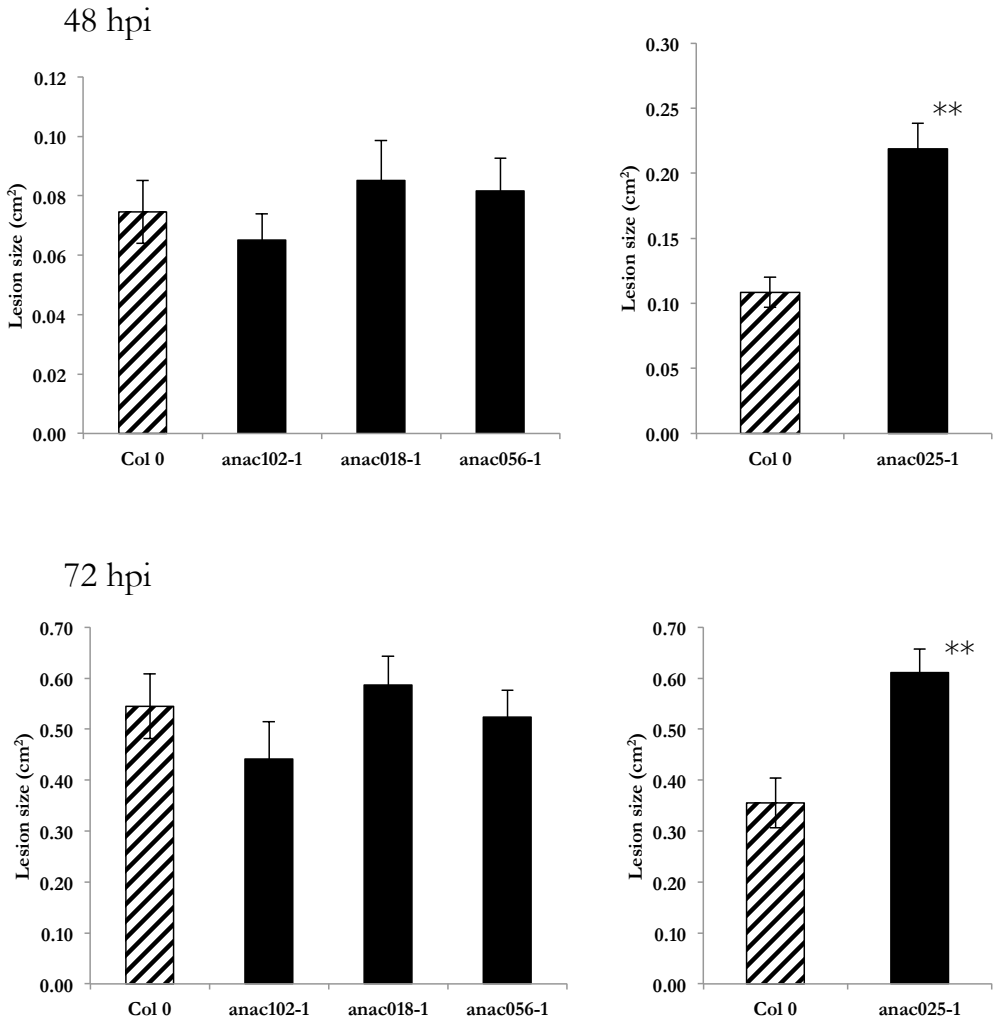


Figure 5.11: **Phenotypes of NAC knockouts during *Botrytis cinerea* infection** Lesion size of *Botrytis* infected Arabidopsis leaves in Col 0 and four NAC knock-out lines at 48 and 72 hpi. *anac018-1, anac056-1* and *anac102-1* were tested in one experiment while *anac025-1* was tested in a separate experiment. Error bars represent SEM (N=30).

Based on lesion size, *anac018-1*, *anac056-1* and *anac102-1* did not show an altered susceptibility to Botrytis phenotype. *anac025-1* showed an increased susceptibility (i.e., lesion size was larger on average) at 48 and 72 hours post infection (figure 5.11). This suggested that *ANAC025* had a role in promoting a protective response to *Botrytis cinerea* infection, which restricts the spread of the lesion. In *anac025-1*, the

response promoted by ANAC025 is diminished and the lesion can spread faster. This phenotype is the opposite to the *ANAC092* knock-out, which could suggest a negative regulation of *ANAC092*. However, the data from the protoplast transactivation assay in the previous chapter suggested a positive role of regulation for ANAC025 on *ANAC092* expression. Similarly, the expression profiles of *ANAC025* and *ANAC092* would be negatively correlated if *ANAC025* had a negative influence on *ANAC092* expression, however they are positively correlated (figure 5.5), suggesting ANAC025 promotes expression of *ANAC092*. Therefore, although it is likely that ANAC025 does positively regulate *ANAC092*, it must also have a role in regulating a number of other genes, expression of which are diminished in *anac025-1*, leading to a more susceptible phenotype.

anac018-1 and *anac056-1* were not observed to show a significant difference in lesion size compared to the wild type at any time point. Reduction in *ANAC102* transcript also did not appear to affect response to *Botrytis cinerea*, despite *ANAC102* being differentially expressed during *Botrytis cinerea* infection. This suggests that these genes do not have a functional role in *Botrytis cinerea* infection, although it is possible they have a subtle, non-phenotypically visible effect. Alternatively, they may be functional homologues of each other and the absence of one protein has a negligible effect due to the presence of a related protein which can act in a similar manner.

ANAC025 is differentially expressed during *Botrytis cinerea* infection and was therefore predicted to have a key role in regulating biotic stress responses. Correspondingly, *anac025-1* demonstrated an increased susceptibility to Botrytis, suggesting that biotic stress response is diminished in *anac025-1.* Meanwhile, *ANAC018* and *ANAC056* were not differentially expressed during Botrytis infection and therefore were not suggested to have a role in regulating responses. *anac018-1* and *anac056-1* did not exhibit an altered phenotype during Botrytis infection, suggesting they are not necessary for observable biotic stress response. Therefore, the mutants of *ANAC025*, *ANAC018* and *ANAC056* all appear to have a phenotype consistent with the predicted function of their respective genes during *Botrytis cinerea* infection.

5.2.3.5. *ANAC092* transgenic lines show an altered phenotype during dark-induced senescence consistent with a positive role in promoting senescence

The rate of dark induced senescence was used to test for senescent phenotypes. Although dark-induced senescence has a number of differences to developmental senescence, it is often a more reliable experimental technique. This is because senescence is a process which can be viewed to gradually occur as the plant develops and therefore timing can deviate between leaves. As such, it is difficult to ensure that senescence is initiated at a similar time across all samples and therefore is comparable. Use

of dark treatment allows senescence to be 'triggered', which means the senescence process is synchronised and therefore can be compared between leaves of the same age on different plants. Senescence was triggered using dark-treatment of rosettes and monitored by R:G ratio of leaf 5.

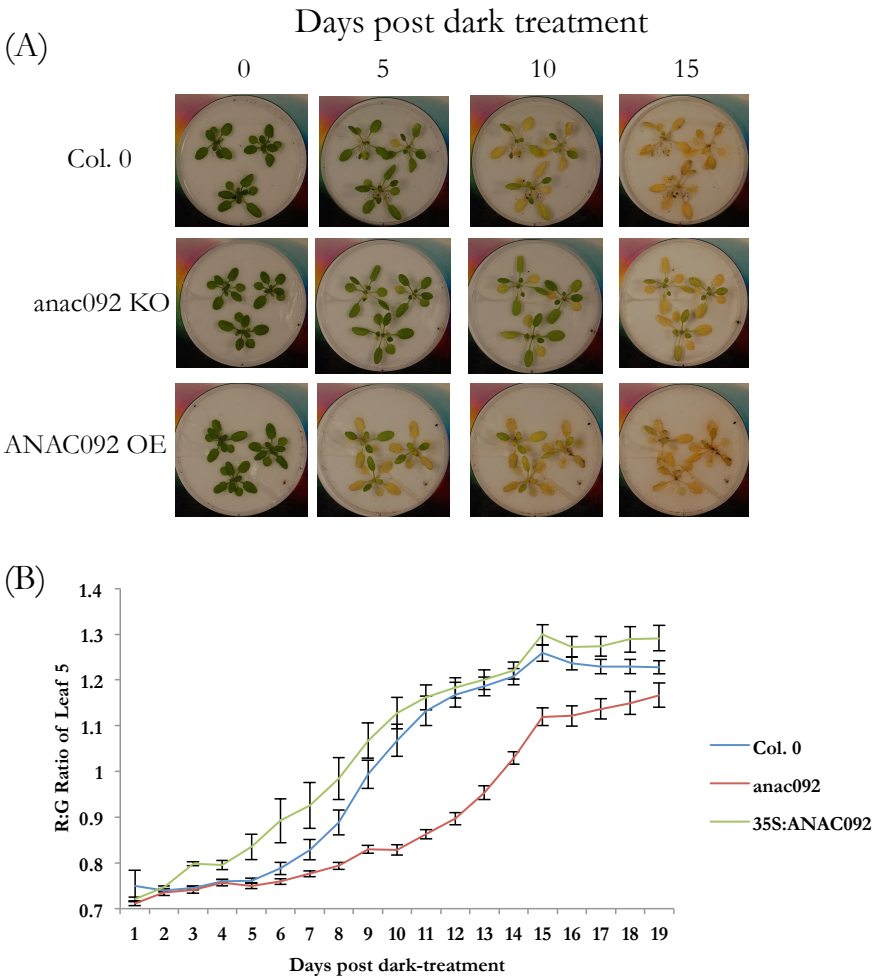


Figure 5.12: **Dark-induced senescence phenotype of *ANAC092* transgenic plants** Progression of dark-induced senescence in *ANAC092* transgenic plants. (A) Images of Col 0, *anac092-1* and 35S:*ANAC092* during dark-treatment, at 0, 5, 10 and 15 days post treatment. (B) Analysis of R:G ratio of leaf 5 over time in *ANAC092* transgenic lines over time, compared to Col. 0 (blue). Visible is a dramatically accelerated phenotype for the *ANAC092* overexpressor (green) and a dramatically reduced phenotype for *anac092-1* (red).

Although dark-induced senescence has a number of differences to age-induced senescence, it is still a closely related form of senescence. As such, many of the processes observed to occur during age-induced senescence occur during dark-induced senescence and many genes retain their original function. As such, it is a useful tool for comparing senescent processes.

The *ANAC092* transgenic lines were tested for a phenotype to confirm ANAC092

conserved its role in promoting senescence during dark-treatment. *anac092-1* demonstrated a delayed senescence phenotype compared to wild type during dark-treatment as is described in Oh *et al.* (1997). Meanwhile senescence was accelerated in the *ANAC092* overexpressor (figure 5.12). This suggests that ANAC092 conserves a role in promoting senescence during dark-treatment.

5.2.3.6. *anac025-1* shows a delayed senescence phenotype during dark-induced senescence

The four NAC knock-out lines were tested for an altered dark-induced senescence phenotype. *anac018-1* and *anac056-1* exhibited a dark-induced senescence phenotype similar to wild type, while *anac025-1* exhibited a delayed senescence phenotype (figure 5.13). In addition, *anac102-1* had a slightly delayed dark-induced senescence phenotype in one screen of two repeats.

This is not consistent with the predictions. *ANAC018*, *ANAC056* and *ANAC102* were differentially expressed during senescence in the timeseries data and therefore were suggested to play a functional role promoting the onset of senescence. Therefore it was expected the mutants of these genes would show an altered rate of senescence. Meanwhile, *ANAC025* was not differentially expressed during senescence, suggesting levels of ANAC025 would be low and therefore *anac025-1* would not exhibit a normal rate of senescence. Instead, *anac018-1, anac056-1* and *anac102-1* did not show a phenotype, while *anac025-1* exhibited delayed senescence. The phenotype of *anac025-1* was similar to the one exhibited by *anac092-1*, which may be consistent with reduced expression of *ANAC092*.

The problem may be that the senescence timeseries data is determined from unstressed plants over time, thus it is developmental senescence as opposed to stress-induced senescence. Dark-induced senescence has a number of similarities to developmental senescence, but also a number of differences (Buchanan-Wollaston *et al.*, 2005). This is because dark-treatment is essentially a form of starvation and therefore a stress. Senescence can be induced by a wide variety of factors, including dark treatment, but each of them will induce its own distinct signalling pathways that will eventually lead to senescence (Guo & Gan, 2011). It is possible that dark-induced senescence triggered a number of stress responses that promote senescence, which did not require signalling through ANAC018, ANAC056 or ANAC102. Meanwhile, the stress response signal activated during dark-treatment does require ANAC025, therefore *anac025-1* shows a phenotype during dark treatment.

It is also possible that ANAC018, ANAC056 and/or ANAC102 are functional homologues of each other. This means that each of the transcription factors could act in an overlapping manner with the other transcription factors. Therefore in any phenotype in the respective mutant lines would be compensated for by the presence of the other transcription factor and as such, mutants would not exhibit a phenotype.

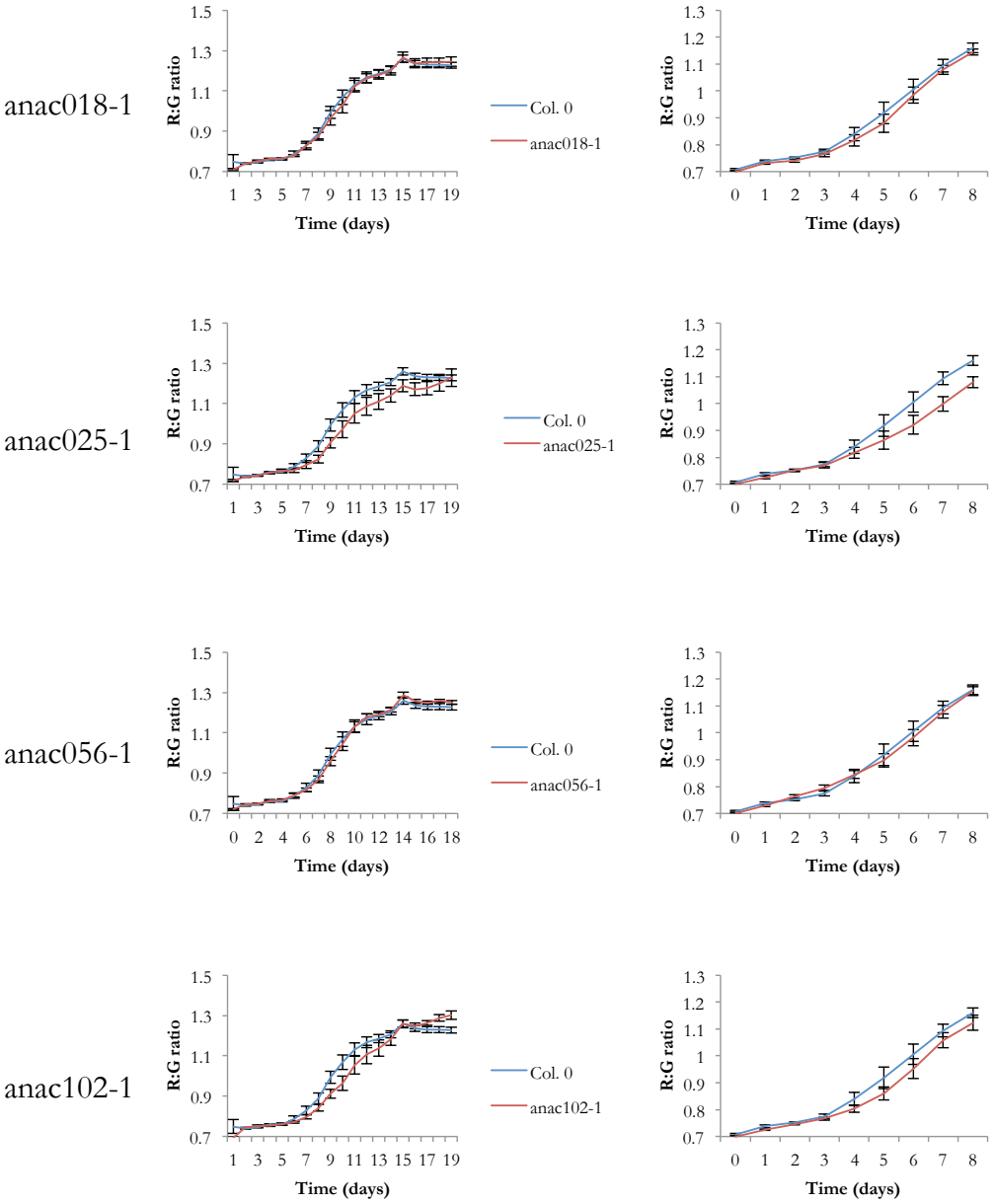


Figure 5.13: **Dark-induced senescence phenotypes of NAC knock-out lines** R:G ratio of leaf 5 in rosettes of Arabidopsis during dark-treatment in two replicate experiments. Each column of graphs represents one complete replicate, while each row represents each T-DNA insertion line (compared to Col 0). R:G ratio of leaf 5 is on the y-axis, while days post treatment is on the x-axis. The NAC T-DNA insertion line is shown in red compared to the wild type Col 0 in blue. Error bars represent SEM (N=9).

5.2.3.7. *ANAC092* expression during *Botrytis cinerea* infection in the four NAC knock-out lines

Phenotypes of a particular mutant can indicate whether expression of that gene is key to a stress response, but this does not show evidence of direct interaction between that gene and *ANAC092* expression. Therefore, *ANAC092* expression was tested in the leaves of the mutant plants during Botrytis infection using qPCR. Originally, 30 hours post Botrytis infection was tested, as this was a maximum point for *ANAC092* transcript levels and it was believed that this time would show the greatest difference between wild-type and the knock-outs.

Leaf 7 from six Arabidopsis plants was detached and infected with *Botrytis cinerea* using drop inoculation method. To confirm *ANAC092* was correctly induced by the treatment, a mock treatment of Col 0 was included.

Levels of *ANAC092* transcript were increased in infected tissue compared to mock treated tissue, suggesting *ANAC092* is increased in expression in response to Botrytis infection (figure 5.14). However, levels of *ANAC092* transcript were not significantly different in the leaves of the four T-DNA insert lines compared to the leaves infected Col 0, suggesting that absence of NAC proteins did not influence *ANAC092* expression during *Botrytis cinerea* infection (figure 5.14).

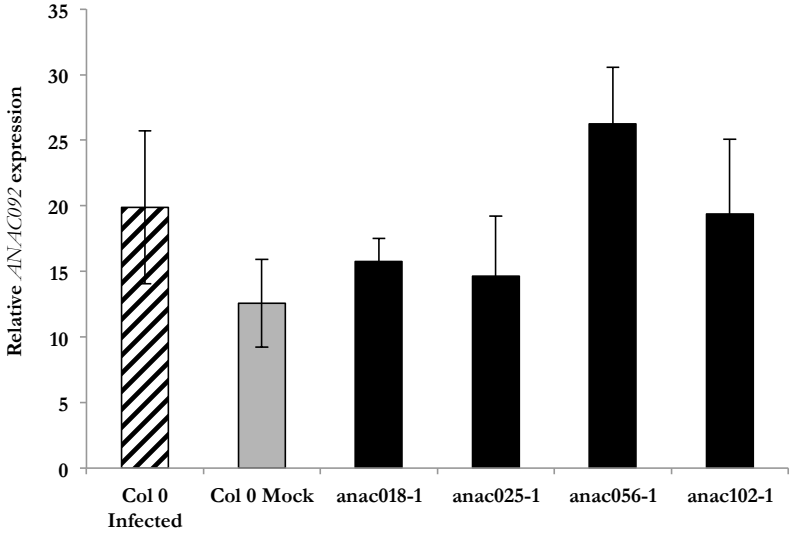


Figure 5.14: **Expression of *ANAC092* in mutant plants at 30 hours post infection with *Botrytis cinerea*** Expression of *ANAC092* during *Botrytis cinerea* infection in Col. 0, *anac018-1*, *anac025-1*, *anac056-1* and *anac102-1* at 30 hours post infection (hpi) as determined by qPCR. Error bars represent SEM (N=6).

A large number of transcription factors were capable of binding to the *ANAC092* promoter in yeast 1-hybrid, therefore it is possible that a redundant mechanism exists to induce *ANAC092* expression in the absence of any one NAC protein. Therefore, it

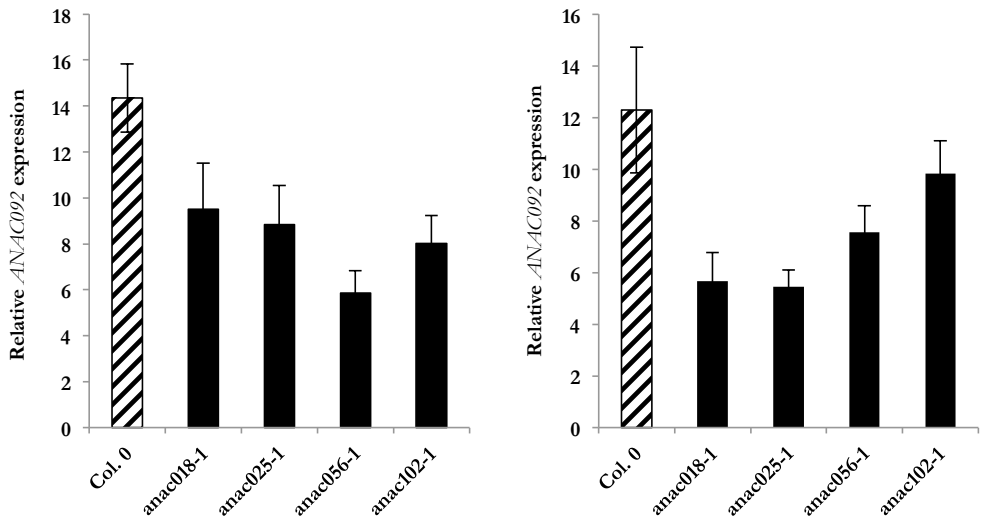


Figure 5.15: **Expression of *ANAC092* in mutant plants at 24 and 28 hours post infection with *Botrytis cinerea*** Expression of *ANAC092* during *Botrytis cinerea* infection in Col 0, *anac018-1*, *anac025-1*, *anac056-1* and *anac102-1* at 24 and 28 hours post infection (hpi) as determined by qPCR. Error bars represent SEM (N=3).

was decided to test *ANAC092* expression at two earlier time points, 24 and 28 hours post infection. This is because it is possible that *ANAC092* may be slightly delayed in initiation of expression, rather then reduced in maximum expression. *ANAC092* expression was tested using qPCR at 24 and 28 hpi (figure 5.15). This did show some differences in transcription levels between the NAC knock-out lines and wild type for *ANAC092* expression. Levels of *ANAC092* transcript were slightly diminished in all NAC knock-outs at both time points (figure 5.15), particularly *anac018-1* and *anac025-1.* However, these results are not at any significant level based on a Student's t-test and so no definitive conclusions can be drawn.

5.2.3.8. ANAC092 expression during dark-induced senescence in the four NAC knock-out lines

During dark-induced senescence, whole rosettes were harvested at 3, 4 and 5 days post treatment. These time points were used as they represented the time where the R:G ratio of Col 0 leaf 5 rose above 7.5. This preceded visible senescent symptoms but it was thought to be important to capture changes in gene expression early as described above. Levels of *ANAC092* mRNA increased from 3 to 5 dpt in Col 0, reflecting the increase in *ANAC092* expression that was expected during dark-induced senescence (figure 5.16). Meanwhile, in the NAC knock-outs, levels of *ANAC092* transcript were similar to Col 0 at all three time points, suggesting the loss of functional NAC genes had little or no effect on *ANAC092* expression during dark-induced senescence.

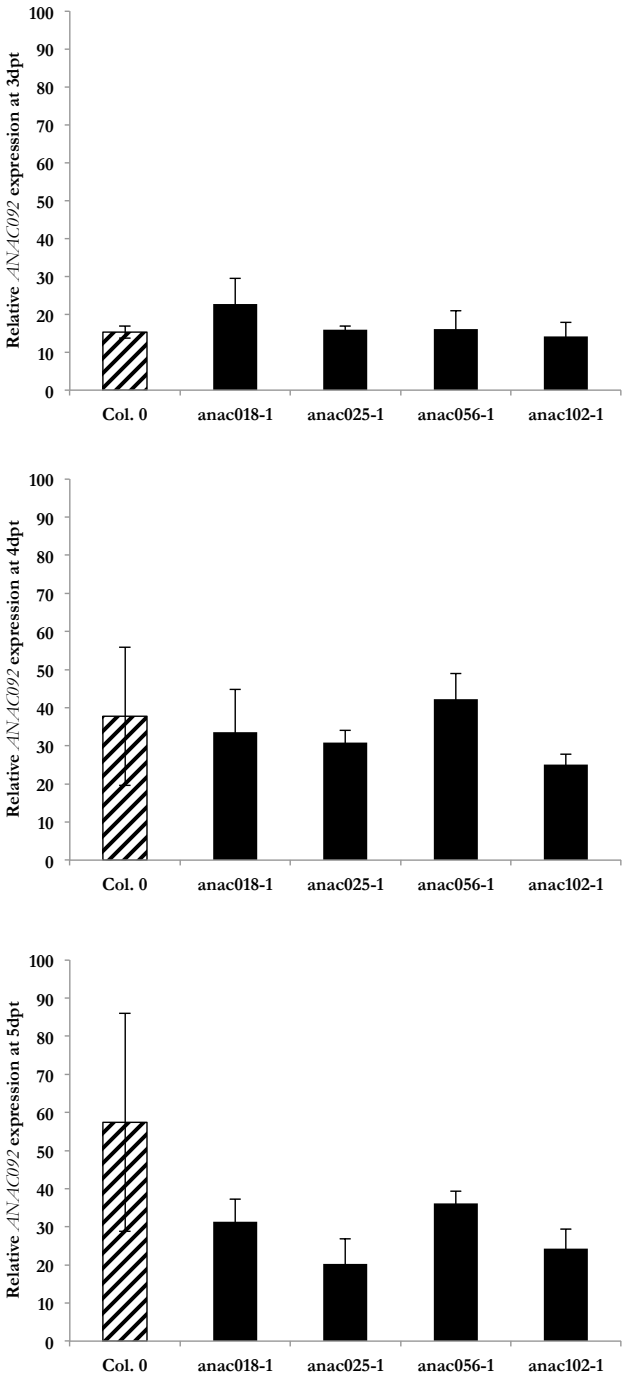


Figure 5.16: **Expression of *ANAC092* in mutant plants during dark-induced senescence** Expression of *ANAC092* during dark-induced senescence in Col 0, *anac018-1*, *anac025-1*, *anac056-1* and *anac102-1* at 3, 4 and 5 days post dark-treatment (dpt) as determined by qPCR. Error bars represent SEM (N=3).

5.2.3.9. Influence of NAC transcription factors on *ANAC092* expression during *Botrytis cinerea* infection and dark-induced senescence

mRNA levels of *ANAC092* appear to be unaffected in the NAC knock-out lines during *Botrytis cinerea* infection or dark-induced senescence. As such, it is not possible to suggest that *ANAC092* levels are dependent on the NAC genes during these stress conditions. It is still possible that they have some function in driving *ANAC092* expression at a very minor level, but has not been detected. They may contribute a very small amount to *ANAC092* expression, which may be detected given more biological replicates which would give a truer representation of a normal distribution. Alternatively, they may be redundant in function with each other, which means the absence of one NAC protein is compensated for by the presence of another. However, in the absence of statistically significant data, it is not possible to reject the null hypothesis and therefore it is not possible to establish whether these NAC genes are necessary for transcription of *ANAC092*.

5.2.3.10. Affect of NAC knock-outs on *ANAC092* transcription compared to predictions

The aim of this chapter was to identify the particular transcription factors that are capable of driving *ANAC092* expression during different stresses. The prediction from PRESTA timeseries data was that ANAC018 and ANAC056 were bound to the *ANAC092* promoter during senescence, while ANAC025 bound to the promoter of *ANAC092* during *Botrytis cinerea* infection. ANAC102 was suggested to bind to the promoter during both conditions.

It was expected to see a diminished expression of *ANAC092* in the appropriate stress for that transcription factor, i.e., in the *anac025-1* line during *Botrytis* infection *ANAC092* transcript would be reduced. In *anac018-1* and *anac056-1* levels of *ANAC092* transcript would be affected during senescence. This was not observed; instead *ANAC092* transcript was not significantly affected in any stress condition or transgenic plant line. Therefore it is not possible to validate the predictions.

5.2.3.11. Different changes in gene expression in *anac025-1* occur during dark-induced senescence and *Botrytis cinerea* infection

It is possible that ANAC025 is not specifically involved in *ANAC092* transcription and response to *Botrytis cinerea*, but instead is a gene that is involved in a range of stress responses. This would make it somewhat similar to *ANAC092*, with a strong presence in the core response network. *anac025-1* is a previously unstudied T-DNA line of Arabidopsis, as such it has no other characterised phenotypes that may highlight a potential role in stress response. To determine functions of *ANAC025* during stress response, global transcriptome changes between Col 0 and the *anac025-1* line during *Botrytis cinerea* infection (24 and 28 hours post infection) and dark

induced senescence (3 & 5 days post treatment) were determined using a NimbleGen 12 × 135k Arabidopsis microarray.

Differential expression of genes was determined as a fold change greater then 2 compared to Col 0. A number of genes were differentially expressed at all time points (table 5.5).

Treatment	hpi/dpt	Fold change < -2	Fold change > 2
Botrytis	24	1586	1241
Botrytis	28	889	1029
Dark Treatment	3	1184	1116
Dark Treatment	5	1631	2178

Table 5.5: **Number of genes differentially expressed in *anac025-1*** Number of genes expressed at fold change > ±2 in *anac025-1* compared to Col 0 as determined by NimbleGen microarray during Botrytis infection (24 and 28 hours post infection) and dark-induced senescence (3 and 5 days post treatment).

If *ANAC025* triggers stress responses during both Botrytis and dark-induced senescence, then genes that were differentially expressed during Botrytis infection and dark-induced senescence could be assumed to be regulated by *ANAC025* in both conditions. Genes that were differentially expressed in *anac025-1* during both Botrytis infection and dark-induced senescence were compared (figure 5.17).

A number of genes were expressed at fold change > ±2 in *anac025-1* compared to Col 0 during two or more time points. The overlap varied between 11% to 25% (figure 5.17).

In order to understand the role of genes targeted by ANAC025, analysis of over-represented GO terms associated with the differentially expressed genes was performed in BiNGO (Maere *et al.*, 2005). For the majority of gene sets, there were no overrepresented GO terms. However, for genes downregulated by 2-fold in *Botrytis cinerea* infection or dark-induced senescence at either time point, a number of GO terms were overrepresented (figure 5.18). There was overrepresentation of GO terms related to cell wall biosynthesis and metabolism (figure 5.17) in the genes downregulated in *anac025-1*. Cell wall biosynthesis and metabolism are important components in pathogen response (Cantu *et al.*, 2008; Lloyd *et al.*, 2011). This suggests *ANAC025* promotes cell wall biosynthesis and metabolism, which is impaired in *anac025-1*. There were no overrepresented GO terms associated with the genes that were upregulated.

Only a very small number of genes were differentially expressed in all conditions. 6 genes were consistently downregulated in both conditions and time points while 13 genes were consistently upregulated in both conditions and time points (table 5.6). Most have not been studied directly, although some have a protein classification

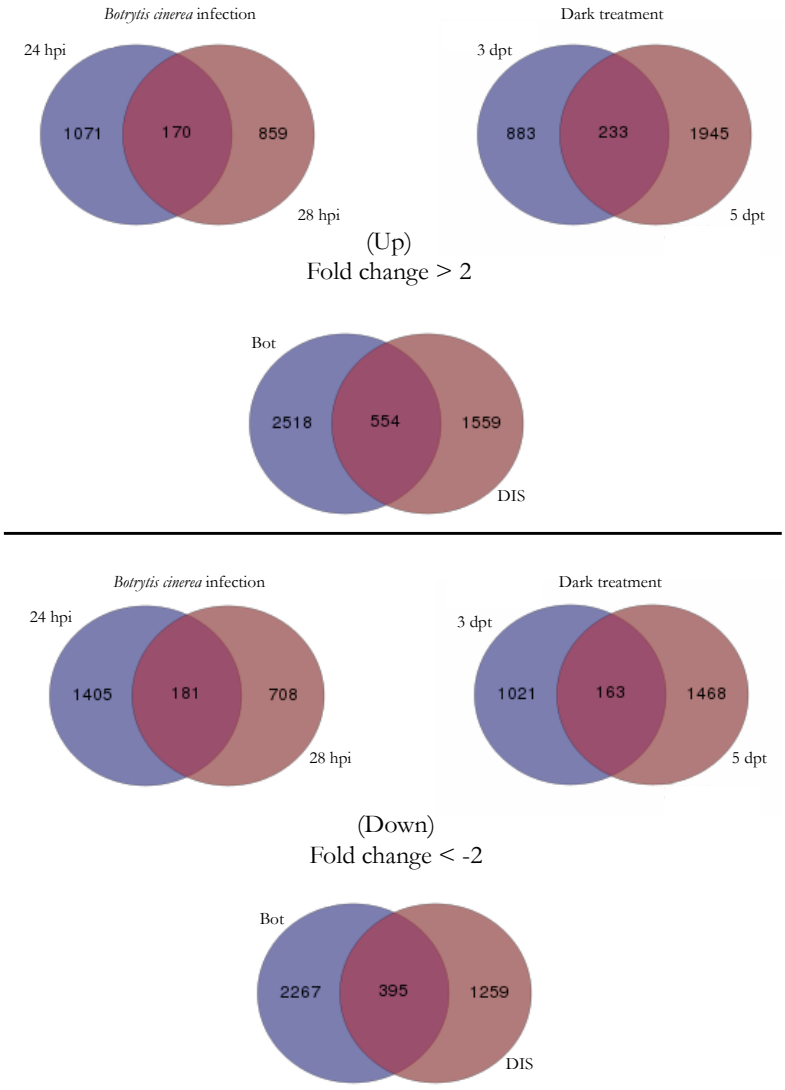
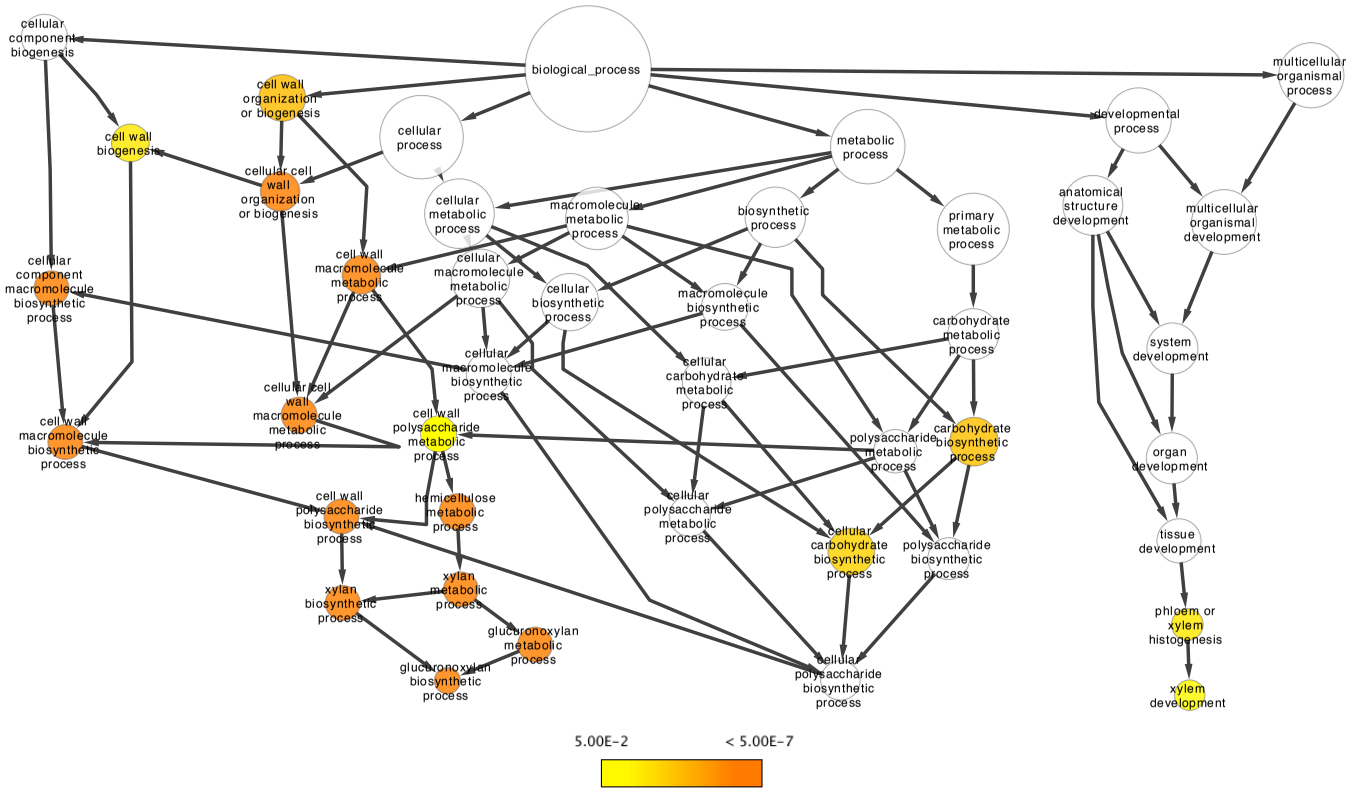


Figure 5.17: **Comparison of genes differentially expressed in *anac025-1* during *Botrytis cinerea* infection and dark-induced senescence** Venn diagram of genes differentially expressed between *anac025-1* and Col 0, during *Botrytis cinerea* infection and dark-induced senescence, when differential expression is defined as fold change $> \pm 2$. The top half of the diagram indicates comparisons between genes that are upregulated, while the bottom half indicates comparisons between genes that are downregulated. In each half, the top two venn diagrams represent the intersect between the two time points within one stress, while the bottom venn diagram indicates the intersect between the two stresses, using every gene that is expressed at FC $> \pm 2$ at either time point.



div

Figure 5.18: **Overrepresentation of GO terms associated with downregulated genes in *anac025-1*** GO terms associated with genes expressed at 2-fold down in *anac025-1* compared to Col 0 in both dark-induced senescence and *Botrytis cinerea* infection at either time point. Colour indicates corrected p-value indicated from a hypergeometric test corrected by a Benjamini and Hochberg correction (Benjamini & Hochberg, 1995).

ATG	Name (TAIR10)	Interpro description	24hpi	28hpi	3dpt	5dpt
AT1G24320	GCS2	Six-hairpin glycosidase-like	-1.55	-1.16	-1.40	-1.14
AT1G51420	SPP1	HAD-like domain	-1.99	-2.55	-1.08	-1.32
AT1G53940	GLIP2	Lipase, GDSL, active site	-1.32	-2.53	-1.17	-2.45
AT3G28899			-5.43	-5.46	-7.92	-4.43
AT4G02810	T5J8.13; T5J8_13	Protein of unknown function DUF3049	-1.27	-1.48	-1.18	-1.12
AT5G65850	K14B20.2; K14B20_2	F-box associated interaction domain	-1.40	-1.40	-1.85	-1.05
AT1G74670	GASA6	Gibberellin regulated protein	2.87	2.15	1.24	1.67
AT2G13550	T10F5.9; T10F5_9		1.04	2.33	3.54	1.87
AT2G39560	F12L6.22; F12L6_22		1.73	2.30	1.28	1.46
AT2G46720	HIC	Thiolase-like	1.37	1.16	1.56	2.33
AT3G05110	T12H1.7; T12H1_7	Domain of unknown function DUF3444	1.40	1.03	2.57	1.20
AT3G06035			1.73	1.10	1.33	1.90
AT3G45440		Protein kinase, ATP binding site	2.90	1.12	2.01	1.53
AT4G04900	RIC10	PAK-box/P21-Rho-binding	2.00	2.02	2.02	2.67
AT5G01740	T20L15.10; T20L15_10	Wound-induced protein, Wun1	2.00	1.21	1.78	1.47
AT5G11440	CID5	Ubiquitin system component Cue	1.46	2.52	1.07	1.83
AT5G28650	WRKY74	Zn-cluster domain	2.35	1.00	5.58	1.96
AT5G46295			6.21	3.95	3.94	4.09
AT5G53680	MGN6.2; MGN6_2	RNA recognition motif domain	1.27	2.27	2.08	1.15

Table 5.6: **Genes consistently altered in expression in *anac025-1* compared to Col 0** Genes expressed with fold change $> \pm 2$ in *anac025-1* compared to Col 0, during *Botrytis cinerea* infection (24 & 28 hpi) and dark-induced senescence (3 and 5 dpt). All gene expression values displayed as $\log_2$ fold change. Protein domains associated with protein sequences of each gene are shown as identified by Interpro scan.

associated with their protein sequence using InterPro scan (Zdobnov & Apweiler, 2001). Protein domains associated with protein sequence of the genes have been noted in table 5.6.

If these genes share ANAC025 as a regulatory mechanism, then they may share a DNA motif in their upstream sequences that ANAC025 recognises. Comparison of the 1000bp upstream promoters of these genes using MEME (Bailey *et al.*, 2009) or Consensus (Hertz & Stormo, 1999) revealed no motif conserved in all sequences. This may suggest that these are indirect targets of ANAC025, i.e. ANAC025 regulates a set of regulatory genes or a biological process, of which these are targets.

Together, these results may indicate ANAC025 has a role in promoting a protective response to *Botrytis cinerea* and promoting dark-induced senescence, shown by the resistant and delayed senescent phenotype of *anac025-1.* The suggestion from gene expression changes in *anac025-1* is that ANAC025 may promote cell wall maintenance during senescence and Botrytis infection. In *anac025-1,* these mechanisms are impaired leading to higher susceptibility to Botrytis and accelerated senescence.

5.2.4. Construction of a multiple tier regulatory network for ANAC092

5.2.4.1. Yeast 1-hybrid of the upstream NAC transcription factors

In an attempt to understand more of the regulatory mechanisms controlling *ANAC092* expression during senescence and *Botrytis cinerea* infection, it was decided to expand the gene regulatory network to incorporate regulatory components separated by an additional transcription factor. The promoter regions for *ANAC018*, *ANAC025* and *ANAC056* were examined for DNA binding using yeast 1-hybrid. Promoter regions have been shown to be conserved in a manner similar to coding regions across many plant species (Baxter *et al.*, 2012), therefore it is possible the promoter regions of these related genes will be conserved in a manner similar to their coding regions.

The 1000bp promoter regions were cloned into three independent ~400bp promoter fragments designed by Peijun Zhang as described in the previous chapter (figure 5.19). Each promoter fragment was screened against the total yeast 1-hybrid library, testing positive interactions twice in a pairwise manner to confirm results.

In total, nine transcription factors were found that bound to the promoter region of *ANAC025* in yeast 1-hybrid. These were from a mix of transcription factor families including ERFs, ATHBs and high-mobility group (HMG) protein. Five transcription factors bound to the *ANAC018* promoter in yeast 1-hybrid, three of which also bound to the *ANAC056* promoter region. This overlap largely consisted of members of the ERF transcription factor family such as ORA59. Therefore, all the proteins that bound to the *ANAC056* promoter region also bound to the *ANAC018* promoter. Although, three transcription factors bound to the *ANAC018* promoter only but not the *ANAC056* promoter region. Eight unique proteins bound to the *ANAC025* promoter but none of these bound to the *ANAC018* or *ANAC056* promoter. Only

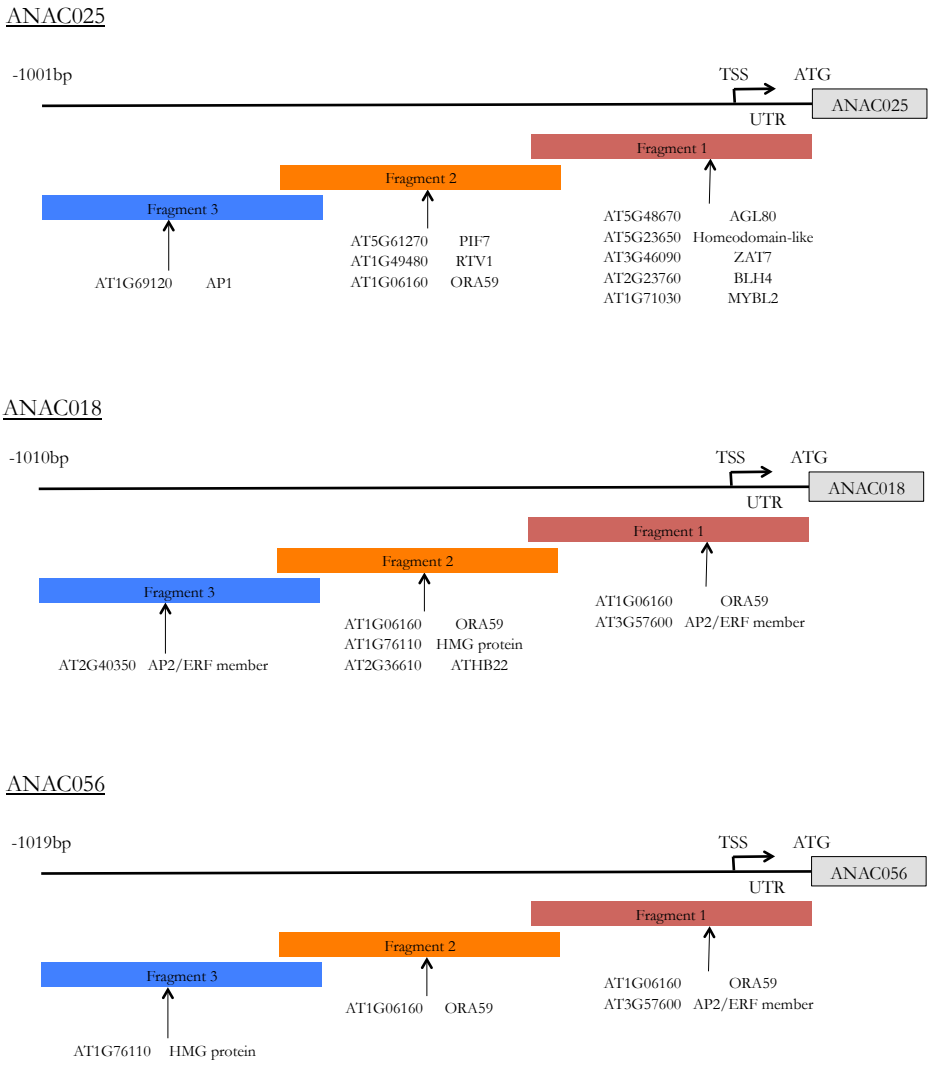


Figure 5.19: **Yeast 1-hybrid results for *ANAC018*, *ANAC025* and *ANAC056*** Yeast 1-hybrid results for the promoter regions of *ANAC025*, *ANAC018* and *ANAC056*, showing each cloned promoter fragment from genomic DNA. Illustrated is each transcription factor that bound to the promoter fragment in pairwise yeast 1-hybrid.

one transcription factor, ORA59, appeared to bind to the promoter region of all three transcription factors.

Interestingly, the yeast 1-hybrid results appear to be indicative of the expression profiles of the target genes (figure 5.20). Nine transcription factors bound to the promoter of *ANAC025* in yeast 1-hybrid. With the exception of ORA59, the yeast 1-hybrid results for *ANAC018* and *ANAC056* were distinct, suggesting different proteins are capable of binding to their promoter regions. These two groups of proteins are expressed under different conditions. ANAC025 is expressed during Botrytis infection while ANAC018 and ANAC056 were expressed during age-induced senes-

cence. The difference in protein:DNA interactions upstream of the coding sequence may account for the differing expression profiles between these transcription factors.

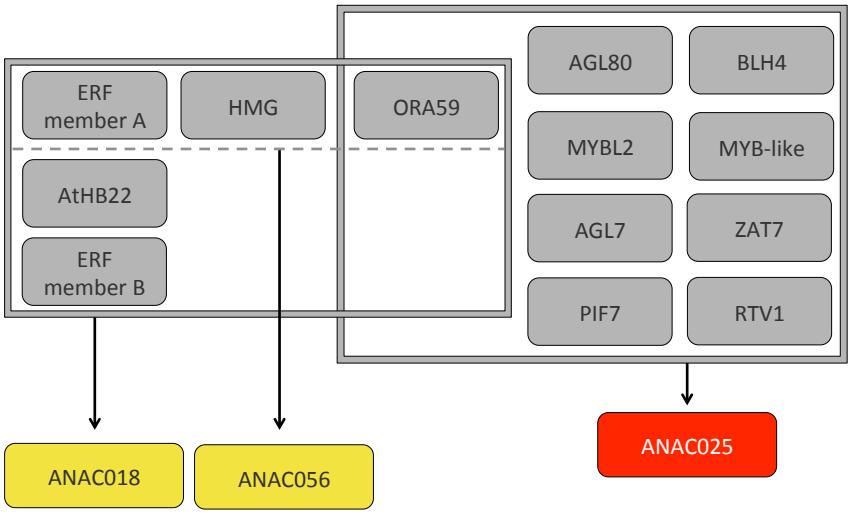


Figure 5.20: **Yeast 1-hybrid results for *ANAC018*, *ANAC025* and *ANAC056* promoter regions** Venn diagram of yeast 1-hybrid results for *ANAC018*, *ANAC056* and *ANAC025*. Each box represents transcription factors that bound to the promoter region. Dotted line indicates transcription factors that bound to promoters of *ANAC056* and *ANAC018*.

ORA59 bound to the promoter region of all three NAC transcription factors in yeast 1-hybrid, in addition to binding to the promoter of *ANAC092* (shown in the previous chapter). ORA59 is a key integrator of jasmonate and ethylene signalling (Pré *et al.*, 2008), therefore it is possible that ORA59 is a 'master regulator' of a large number of genes downstream of these signalling pathways. However, none of these transcription factors were differentially expressed in results using an inducible expressor of *ORA59* (Pré *et al.*, 2008), suggesting that increased ORA59 levels are not capable of driving their expression. Perhaps, ORA59 has a high propensity for binding to DNA, but does not demonstrate strong transcriptional activity until other factors such as heterodimerisation or post-translational modifications are active. Alternatively, ORA59 is highly active in yeast 1-hybrid and may produce a number of false positive results. This could be investigated with a negative control promoter region composed of a random sequence, which should not show binding for any transcription factor.

5.2.4.2. Network constructed from Y1H interactions determined by the PRESTA group

As part of the PRESTA project, a large number of yeast 1-hybrid interactions have been completed creating a network of protein-DNA interactions that may be responsible for a number of stress responsive pathways. A number of these can suggest interactions between genes that may be directly or indirectly related to expression of *ANAC092*. A network of yeast 1-hybrid interactions was generated from data previously presented in this work as well as additional data generated as part of the PRESTA project (figure 5.21). These yeast 1-hybrid interactions were all validated by three individual pairwise repeats as in the previous chapter. This network has a certain bias towards genes that are effective in yeast 1-hybrid but may identify interactions that explain biologically significant regulatory pathways.

5.2.4.3. A number of ERF proteins make a smaller network in the larger ANAC092 network

In the yeast 1-hybrid network, a group of ethylene responsive genes such as ERF1, ERF4 and ATERF15 bound to the upstream genomic region of *ORA59*, suggesting an ethylene driven process of expression (figure 5.21). ERF1 and ERF14 bound to the promoters of *ERF15* and *ORA59*, which also bound to the upstream regions of each other. These ERFs are highly related, being members of the same B-3 ERF/AP2 family (Nakano *et al.*, 2006). In addition, ORA59 and ATERF15 are the most closely related proteins to each other, sharing 63% amino acid composition (Pré *et al.*, 2008). This suggests that the highly related ERF proteins form an internal network that allows them to regulate each other, similar to the way WRKY proteins have been proposed to do (Dong *et al.*, 2003; Eulgem & Somssich, 2007). In the *ANAC092* yeast 1-hybrid network, these ERF proteins may represent an ethylene signalling pathway that transmits ethylene signals to *ANAC092*, through the action of ORA59 on the *ANAC092* promoter region.

5.2.4.4. GBF1 and PIF7 may represent a light or oxidative stress signalling component in the ANAC092 regulatory network

GBF1 and PIF7 are transcription factors related to light signalling. GBF1 is a well characterised protein shown to bind to the G-Box motif (CACGTG) in light responsive genes after phosphorylation (Klimczak *et al.*, 1992; Schindler *et al.*, 1992a,b). GBF1 appears to be a transmitter of blue light response signalling (Mallappa *et al.*, 2006) and is degraded in dark-treated seedlings (Mallappa *et al.*, 2008). Interestingly, GBF1 has been shown to positively regulate senescence during oxidative stress through negatively regulating the *CAT2* gene (Smykowski *et al.*, 2010). In yeast 1-hybrid analysis, GBF1 bound to the promoter of *ANAC025*, so it is possible that GBF1 indirectly regulates *ANAC092* through *ANAC025.* Senescence and salt stress

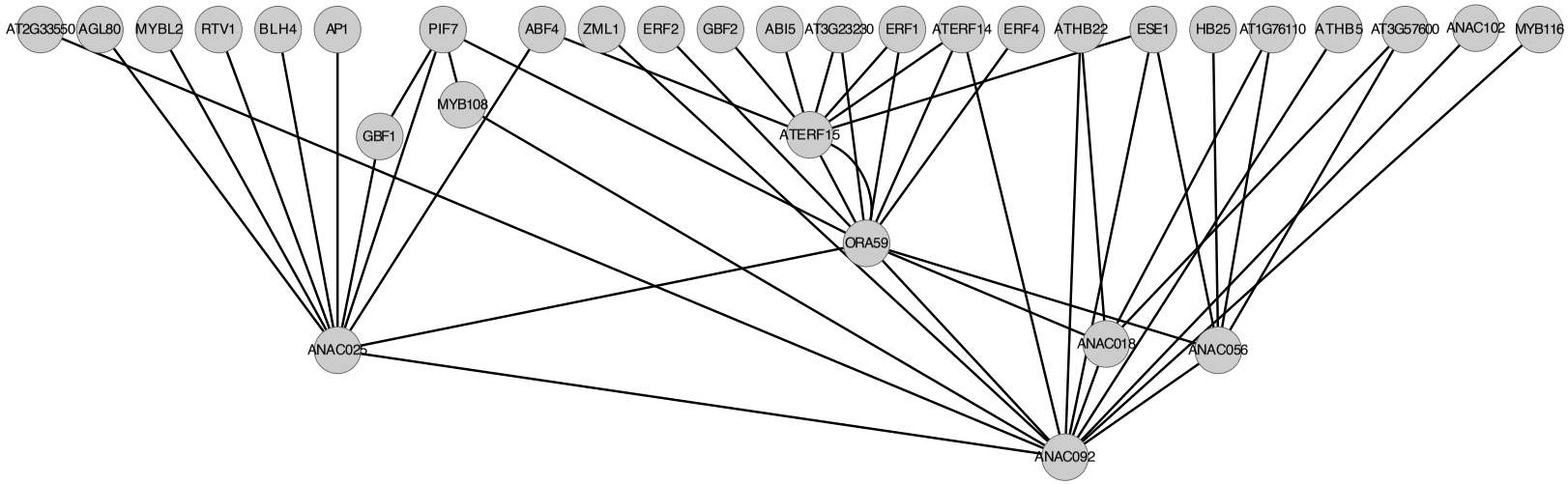


Figure 5.21: **Yeast 1-hybrid network for *ANAC092*** Network of yeast 1-hybrid interactions upstream from *ANAC092*. Edges are directed downwards, with the top node representing the transcription factor binding to the promoter of the gene below it. The only exception is ORA59 binding to the promoter of *ATERF15*.

are linked through H_2O_2 based signalling (Allu *et al.*, 2014) and ANAC092 functions in both response to high-salinity and oxidative stress (Kim *et al.*, 2011). This suggests that *ANAC092* may be linked to oxidative stress responses indirectly via *GBF1*.

PIF7 is a member of the Phythochrome Interacting Factor (PIF) transcription factors, a group of basic helix-loop-helix (bHLH) proteins that respond to light (Duek & Fankhauser, 2005). PIF7 is known to bind to the promoter of *DREB1C* and represses expression in the presence of light through a G-box interaction (Kidokoro *et al.*, 2009). PIF7 accumulates in the shade in a non-phosphorylated form and activates auxin responsive genes, therefore driving growth (Li *et al.*, 2012). Like other PIFs, PIF7 rapidly migrates to the nucleus upon activation of the phytochrome phyB, but unlike other PIFs is not degraded shortly after activation (Leivar *et al.*, 2008). A model exists for PIF7 where the phosphorylated, inactive form is transported to the nucleus after binding to Pfr (phyB which is activated in light), while in the shade phyB becomes inactive (known as Pr) and migrates out of the nucleus, leaving PIF7 there. PIF7 then rapidly becomes dephosphorylated and drives expression of auxin biosynthesis genes (Li *et al.*, 2012). In the *ANAC092* yeast 1-hybrid network, PIF7 bound to the promoter region of *GBF1*, *MYB108* and *ANAC025*, suggesting it may be a central regulator in stress response signalling upstream from *ANAC092* and may indirectly regulate *ANAC092* in response to stress. GBF1 and PIF7 may represent a previously uncharacterised link between light conditions, oxidative stress and *ANAC092* induction, which in turn may regulate senescence processes.

5.2.4.5. 'Feed-forward' mechanisms present in the ANAC092 regulatory network may impart temporal dynamics of ANAC092 expression

A number of 'feed-forward' mechanisms appear to exist in the network. For example ESE1 bound to the promoter of *ANAC056* and *ANAC092*, suggesting it regulates both *ANAC092* and *ANAC056*, which in turn also regulates *ANAC092* (figure 5.21). Feed-forward mechanisms appear to confer increased dynamic regulation based on the topology of the feed-forward mechanism (Mangan & Alon, 2003). 'Coherent' feed-forward loops, where the indirect interaction matches the direct interaction, seem to impart a slight delay after input, while 'incoherent' feed-forward loops generate a rapid response to input stimulus (Mangan & Alon, 2003). It is possible that a number of these systems exist to finely tune the dynamics of *ANAC092* expression during stress with respect to time.

5.2.4.6. hCSI modelling applied to the ANAC092 network suggests a number of regulatory pathways

Previously, hCSI modeling was used to analyse the influence a particular transcription factor may have in regulating *ANAC092* expression during *Botrytis cinerea* in-

fection and dark-induced senescence from timeseries expression data. This principle can be used on a larger network which may elucidate roles for multiple interactions on a larger scale. The hCSI algorithm was used for each edge in the network, using the PRESTA timeseries data for developmental senescence and Botrytis infection. This generated a number of small networks specific to each target gene, which were then amalgamated to form a much larger network (figure 5.22).

The network illustrated a number of interactions that may be biologically relevant. Unlike the hCSI modelling applied specifically to *ANAC092,* marginal probabilities reached highs of 1.0 (maximum). Previously the highest marginal probability was 0.26. This indicates the algorithm is predicting the interaction more often, therefore it is granting a stronger weighting to the interaction. It is possible that the algorithm is not appropriate for predicting the expression for *ANAC092*, but is suitable for other genes in this network. As such, the algorithm was effective at predicting the regulatory networks for the other transcription factors, but not for *ANAC092.* Exactly why this is the case is unclear.

5.2.4.7. PIF7 is predicted to regulate *GBF1* and *MYB108* in both senescence and during Botrytis infection

Previously, it was suggested that PIF7 and GBF1 may form some component of light signalling that was linked to *ANAC092* expression. PIF7 bound to the promoter regions of *GBF1*, *MYB108* and *ORA59.* hCSI predicted that *PIF7* would regulate *GBF1* and *MYB108* in both stress conditions. This perhaps suggests a link between these three transcription factors which will then regulate *ANACO25* and/or *ANAC092*. Both *GBF1* and *MYB108* are associated with oxidative stress signals, but have been analysed in different contexts. The *MYB108* T-DNA line *bos1* induces increased ROS production during *Botrytis cinerea* infection and wounding, causing enhanced cell death (Mengiste *et al.*, 2003; Kraepiel *et al.*, 2011; Cui *et al.*, 2013). *GBF1* appears to negatively regulate *CAT2*, an enzyme responsible for removal of H_2O_2. Therefore *GBF1* seems to downregulate clearance mechanisms for reactive species and allow accumulation which induces senescence (Smykowski *et al.*, 2010). Perhaps *PIF7* represents a common induction mechanism for these two, that may link light responses in addition to other signals.

PIF7 also bound to the promoter of *ORA59*, the known regulator of *Botrytis* response. Interestingly, while PIF7 was predicted to regulate *MYB108* and *GBF1* equally for both senescence and *Botrytis* infection, PIF7 was predicted to regulate *ORA59* specifically during senescence (figure 5.22). *ORA59* is differentially expressed during developmental senescence, but no role for *ORA59* during senescence has been identified.

A T-DNA insert line for PIF7, previously used in Leivar *et al.* (2008), SALK-062756, was studied for Botrytis susceptibility and dark-induced senescence pheno-

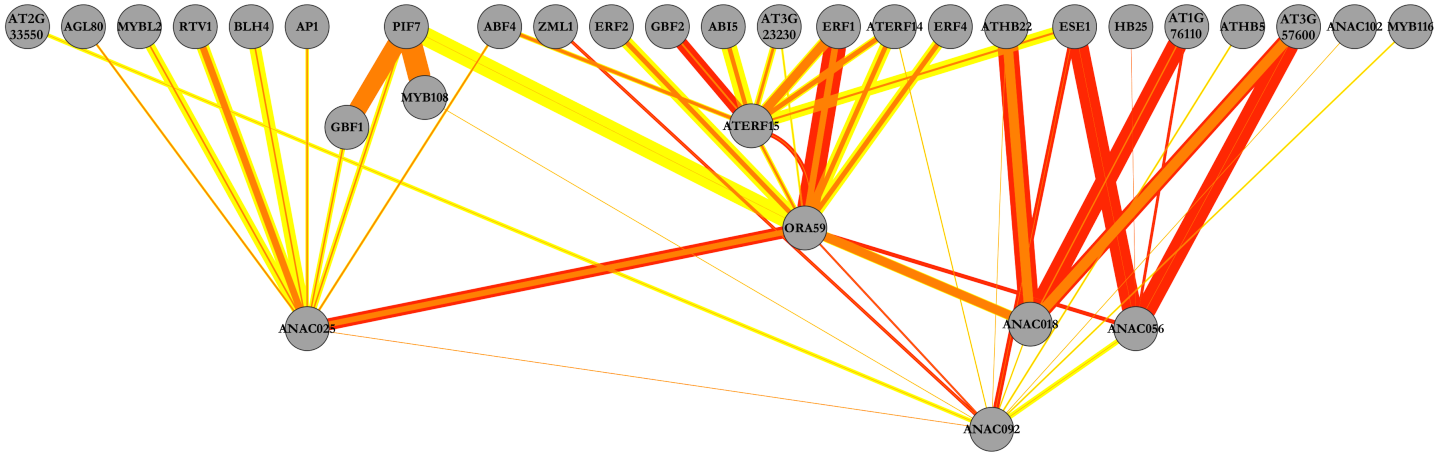


Figure 5.22: **Regulatory network for *ANAC092* predicted by hCSI** Network shown in figure 5.21 with the additional illustration of marginal probabilities determined by hCSI. Red represents *Botrytis cinerea* infection, while yellow indicates long day senescence, orange implies the presence of both. The width of the edge is proportional to the marginal probability, with thickest representing 1 and thinnest representing 0. Like in figure 5.21, each edge points downwards, with regulator at the top and target at the bottom.

type. The PIF7 knock-out was slightly more resistant to *Botrytis cinerea* infection and had a slightly delayed dark-induced senescence phenotype (figure 5.23). This suggests that PIF7 promotes stress responses which are diminished in the knock-out. Whether these function through *GBF1*, *MYB108* or *ORA59* can not be identified without further study, but it does indicate that PIF7 may have a role in different stress responses.

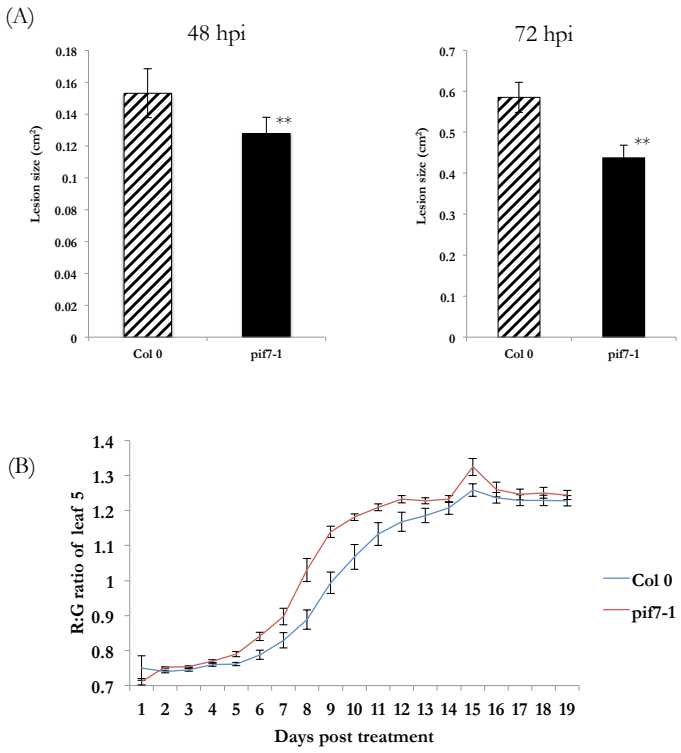


Figure 5.23: **Phenotype of PIF7 knockout line during *Botrytis cinerea* infection and dark-induced senescence** (A) *pif7-1* has smaller lesions at 48 and 72 hours post infection compared to Col 0. (B) *pif7-1* also has a delayed senescent phenotype during dark-induced senescence, as monitored by R:G ratio of leaf 5.

5.2.4.8. ESE1 may coregulate *ANAC018*, *ANAC056* and *ANAC092*

The transcription factor ESE1 recognised the promoter regions of *ANAC018*, *ANAC056* and *ANAC092* in yeast 1-hybrid. Furthermore, it was predicted to regulate *ANAC018* and *ANAC056* during *Botrytis cinerea* infection. ESE1 is known to transmit ethylene responsive signals during salt-stress (Zhang *et al.*, 2011), therefore it is possible ESE1 regulates the expression of these NAC genes during stress conditions in an ethylene responsive manner. To test this, the *ESE1* T-DNA insert line SALK-128736, used in Zhang *et al.* (2011), was tested for a phenotype during *Botrytis cinerea* infection and dark-induced senescence. *ese1-1* did not show a phenotype during *Botrytis cinerea* infection or dark-induced senescence (figure 5.24), suggesting it is not essential for

stress responses or an alternative protein of redundant function compensates for the *ESE1* mutation. This may indicate that while ESE1 is predicted to regulate expression of the NAC genes, it may not be obligatory due to the action of redundant transcription factors.

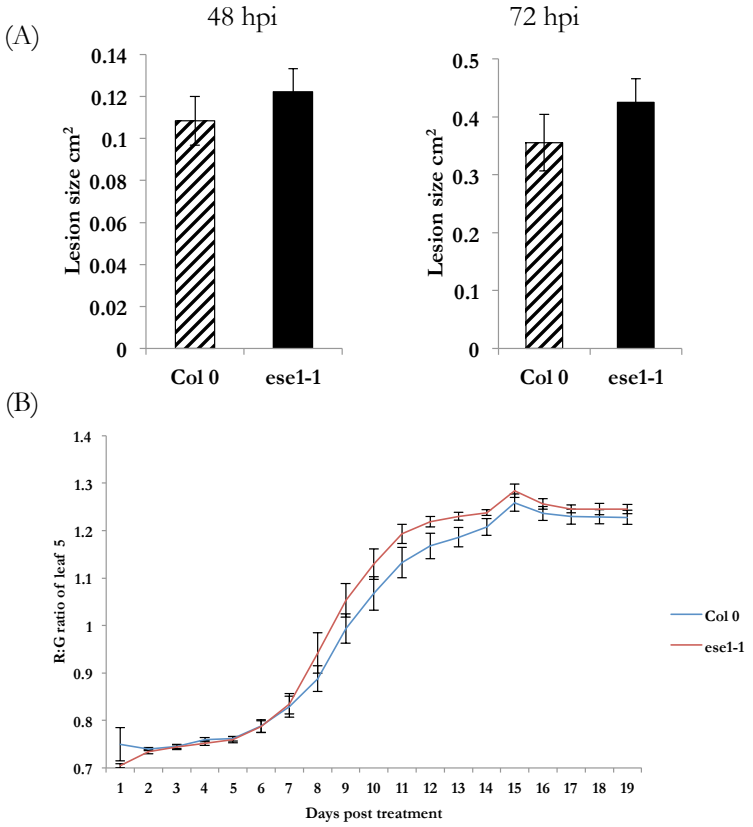


Figure 5.24: **Phenotypes of ese1-1 (SALK-128736) during *Botrytis cinerea* infection and dark-induced senescence** (A) Lesion size of *ese1-1* at 48 and 72 hours post infection with *Botrytis cinerea* (B) R:G ratio of leaf 5 of *ese1-1* and Col 0 during dark-treatment.

5.2.4.9. *ORA59* is a major hub in the ANAC092 regulatory network

Both hCSI and yeast 1-hybrid analysis have emphasised that *ORA59* could act as a major hub in multiple stress responses. In yeast 1-hybrid it binds to the promoter region of *ANAC018*, *ANAC025*, *ANAC056* and *ANAC092*, suggesting a central role in the induction of NAC gene expression. A number of ERF proteins bound to the promoter of ORA59, correlating with its known role in promoting ethylene responses (Pré *et al.*, 2008). hCSI predicts ORA59 as a major regulator of expression of *ANAC018*, *ANAC025* and *ANAC056* during Botrytis infection. Together, this suggests that ORA59 is a central hub in the stress response network, by coregulating a number of stress responsive transcription factors.

5.3. Discussion

5.3.1. Timeseries data contributes to studying gene regulatory networks

Frequently, when a number of potential transcription factors are identified as potential regulators of a gene, the first step is to review literature to determine previously known roles of these proteins. This is then used to guide future research, by establishing the context in which that particular transcription factor may regulate the target protein. However, this methodology is biased towards characterised genes and may miss key attributes of less-studied genes that are important. Being able to infer function of genes from alternative whole genome data sources allows characterisation of genes that are previously unstudied in a particular condition and can be used to demonstrate novel function.

The high degree of coverage offered by PRESTA timeseries microarrays means an expression profile during *Botrytis cinerea* infection and age-induced senescence was available for every transcription factor that bound to the promoter of *ANAC092* in yeast 1-hybrid. The timing of gene expression is extremely important when studying gene regulatory networks, as it is the relationships between temporal expression patterns that allows us to reverse engineer gene regulatory networks. Therefore, timeseries data is a valuable asset when studying networks of transcription factors.

Timeseries data comes with limitations however. Although microarrays have dropped in price significantly, the cost and labour involved in conducting high resolution timeseries is still prohibitive. Furthermore, computational analysis for timeseries data is more complicated and time consuming compared to single timepoint experiments.

To compound this, the quality of predictions is affected by the resolution of the timeseries data. The timeseries data for developmental senescence was taken at a resolution of one timepoint per two days, compared to the *Botrytis cinerea* data which was a higher resolution of one time point every two hours. In developmental senescence, the unstudied period between a gene could be a full 48 hours, which is certainly long enough for a gene to be upregulated and regulate a target gene before the next timepoint. This effect is reduced in the *Botrytis cinerea* infection experiment, with only 2 hours between each time point, however it is still possible that a large number of regulatory steps are occurring within each of these periods.

There are still limits to what gene expression data can tell us. A gene can be induced in response to a stress, but it does not tell us if it is an active protein. The protein is not necessarily synthesised in a functional form and may be post-translationally modified to induce activity. In this situation, the gene expression level of a transcription factor will be relatively stable across the time course, but the level of functional protein will be dynamically fluctuating.

Similarly, it is presumed that a transcription factor will primarily function through

transcriptional activity, thus inducing or repressing the gene expression of downstream targets, but some transcription factors have been identified to function through protein:protein interactions (Rauf *et al.*, 2013). In this situation, alterations in activity of the transcription factor will not be detectable in the transcriptome.

5.3.1.1. hCSI as a network inference modelling technique

In this chapter, the modelling technique hCSI (hierarchical causal structure identification) was used to define regulatory subnetworks within a strictly *in vitro* yeast 1-hybrid network. First, it was used to predict whether transcription factors that bound to the promoter of ANAC092 in yeast 1-hybrid could act during *Botrytis cinerea* infection or age-induced senescence, based on their gene expression profiles. Later, it was used to predict functional interactions of a larger multilayered yeast 1-hybrid network during *Botrytis cinerea* infection and age-induced senescence. CSI works by using the regulator timeseries data to 'predict' the target timeseries data, which can then be tested for accuracy against the known target gene expression profile (Klemm, 2008). The original CSI (Klemm, 2008) allowed the use of multiple datasets to predict a single network, however the development by Penfold *et al.* (2012) to a hierarchical form allows underlying subnetworks specific to each dataset to be determined and compared. This hierarchical development was utilised to predict which edges in the network are ubiquitous to multiple stresses and which are specific to a single stress.

For the immediate upstream transcription factors of ANAC092, hCSI produced low marginal probabilities for all potential regulators. The highest marginal probability was 0.25, representing a 25% confidence that the transcription factor regulated ANAC092 expression. This is a low number of predictions, compared to the >0.95 marginal probabilities generated later in the project for the larger network. It is difficult to determine why this may be, although it is presumably a product of the expression data of *ANAC092*, as this is the only data conserved between both stresses and regulators during modelling. An appropriate positive control of *ANAC092* regulation such as EIN3 (Li *et al.*, 2013; Kim *et al.*, 2014) may help determine the successfulness of a modelling technique, however EIN3 is activity is promoted through post translational interaction with EBF1 and EBF2, generated by EIN2 (Guo & Ecker, 2003; Potuschak *et al.*, 2003; Binder *et al.*, 2007; Cho *et al.*, 2012), which would not be included in the hCSI datasets.

An example of the low marginal probability and timeseries data that has been used to generate the prediction is given in figure 5.25. The expression profile of *ESE1* is shown, which gave the highest marginal probability using the *Botrytis cinerea* data of any transcription factor that bound to the promoter of *ANAC092* (0.257). Also shown is the expression profile of *ATERF14*, which gave a relatively low marginal probability of 0.009. By eye, it appears *ANAC092* may be regulated by both *ESE1*

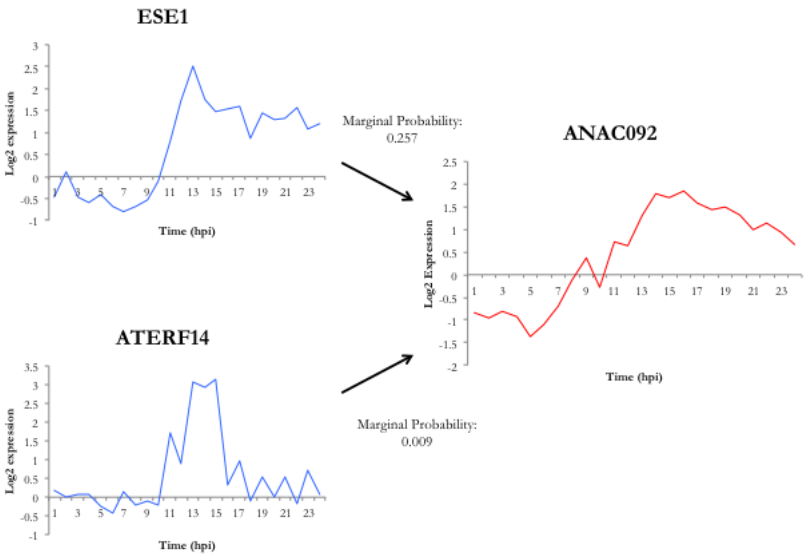


Figure 5.25: **Evaluation of hCSI algorithm** Expression profiles during infection by *Botrytis cinerea* for *ESE1*, *ATERF14* and *ANAC092*. Also shown are marginal probabilities generated by hCSI for *ESE1* and *ATERF14* regulating *ANAC092*.

and *ATERF14* as there is a dramatic increase in expression for both genes just prior to *ANAC092* expression, however hCSI has produced dramatically different predictions for each transcription factor. This is possibly because hCSI looks for smooth non-linear functions between the two expression profiles, which means that two profiles that reflect each other are more likely to be predicted to regulate one another. *ANAC092* has an expression profile that is more similar to *ESE1* then *ATERF14*, because *ATERF14* expression drops off shortly after it is induced while *ESE1* and *ANAC092* remain at high levels after being induced. This means hCSI rejected the influence of *ATERF14* on *ANAC092* more than the influence of *ESE1* on *ANAC092* expression.

The comparison of these expression profiles alludes to a broader issue in transcription. Does expression depend on the continuous action of transcription factors to maintain transcription, or does a momentary activation create an increase in transcription rates that is subsequently sustained? In this example, is maintenance of *ESE1* expression required for *ANAC092* transcription or can expression of the regulator decrease and the presence of residual protein allow for continued transcription? Alternatively, is transcription activated by the presence of appropriate transcription factors, before being maintained regardless of the presence of those initial transcription factors. Explaining facets of transcription such as this would go some way to better explaining the relationship between expression curves of regulators and targets. In turn, this may explain how some network inference algorithms based

on transcription are more successful than others, in addition to directing how new network inference techniques are constructed.

For the larger network, predictions were more varied and showed values from 1 to 0. This may indicate the algorithm was better suited to other gene expression profiles. This data was used to infer indirect regulatory pathways upstream of *ANAC092* that may be important for its expression.

In the network, certain transcription factors appeared as 'hubs', such as *PIF7* and *ORA59*. A number of transcription factors recognised their promoter regions in yeast 1-hybrid. In turn, the PIF7 and ORA59 proteins recognised a large number of promoter regions in yeast 1-hybrid, suggesting these genes are highly connected in the stress response networks. Gene regulatory networks often follow a 'scale-free' pattern, where a small number of nodes are highly connected to other nodes in the network. In a gene regulatory network these nodes will be both regulated by and regulate a large number of genes, causing them to be central to the topology of the gene regulatory network (Albert, 2005). These highly connected nodes are often 'master regulators', conferring a switch from one state to another. This has been commonly studied in plant developmental biology, where a single or small sample of proteins will trigger the induction of a large scale transition in developmental stages (Kaufmann *et al.*, 2010).

The 'hub' genes in the *ANAC092* network were also predicted to regulate many other downstream genes in hCSI, such as PIF7 which was predicted to regulate expression of both *GBF1* and *MYB108* during *Botrytis cinerea* infection and age-induced senescence. This strong prediction may suggest these genes are central to the regulatory network, therefore they are critical for a correct and proper plant immune response. In this way, they may act as 'master regulators' described above.

Interestingly, the genes predicted to regulate *ANAC092* during senescence appeared to have more genes predicted to regulate them during *Botrytis cinerea* infection. For example, ANAC056 was predicted to regulate *ANAC092* during developmental senescence in hCSI, but the majority of the genes upstream were predicted to regulate *ANAC056* during *Botrytis cinerea* infection. This may be an artifact of the minimal change in expression during *Botrytis cinerea* infection, i.e., if the regulator and target gene have relatively flat expression profiles during *Botrytis cinerea* infection, it is more likely the algorithm will predict the regulator influences the target gene expression.

5.3.1.2. Evaluation of predictions compared to data from mutant plant lines

Use of the timeseries data to infer the regulatory network of *ANAC092* expression allowed identification of potential regulators of *ANAC092* during *Botrytis cinerea* infection and age-induced senescence. A number of regulators that may bind to the *ANAC092* promoter during *Botrytis cinerea* infection were identified including the

known biotic stress response genes MYB108, ATERF14 and ORA59. In addition, a number of genes not previously known to have a role in biotic stress were suggested to bind to the *ANAC092* promoter during *Botrytis* infection including ANAC025 and ESE1. Of these, MYB108 did appear to be involved in *ANAC092* expression during *Botrytis cinerea* infection shown by decrease of *ANAC092* expression in *myb108-1*. This data supports the hypothesis that MYB108 promotes *ANAC092* expression during Botrytis infection, as suggested by the predictions. Similarly, ANAC025 appears to have a role in biotic stress, as shown by a more susceptible phenotype to *Botrytis cinerea* infection in ANAC025 mutants. *ANAC092* was not significantly reduced in expression in *anac025-1,* suggesting that ANAC025 is not essential for *ANAC092* expression during *Botrytis cinerea* infection, although it may contribute.

Predicting which genes regulated ANAC092 during age-induced senescence was not as successful as predictions for Botrytis infection. A number of genes were predicted to regulate *ANAC092* during developmental senescence, including ANAC018, ANAC056, ORA59 and MYB108. As with *Botrytis cinerea* infection, *myb108-1* showed a phenotype during senescence and expression of *ANAC092* was reduced in *myb108-1*, congruent with predictions. In contrast, *anac018-1* and *anac056-1* mutants did not display any phenotype and *ANAC092* expression was not significantly affected during dark-induced senescence. This may be due to ANAC018 and ANAC056 acting as functional homologues of each other, therefore complementing the mutant line of the other protein. As such a double, or even triple mutant of these NAC genes may have a more pronounced effect.

Therefore only a small number of predictions were validated. However, it may be a function of the testing methodology used that meant the results were not consistent with the predictions.

5.3.1.3. Dark-induced senescence is a stress and has differences to developmental senescence

Studying age-induced senescence is inherently difficult due to the timescale and large amount of variation that occurs even in genetically identical populations. It is possibly for this reason that no genes in the list of potential transcription factors that regulate *ANAC092* had been studied for developmental senescence. Dark-induced senescence is often used as a proxy for developmental senescence, but has a number of fundamental differences (Buchanan-Wollaston *et al.*, 2005). Perhaps the most important is that dark-treatment is a stress, as such a number of different regulatory genes are expressed (Lin & Wu, 2004; Buchanan-Wollaston *et al.*, 2005). ANAC025 was predicted to regulate *ANAC092* during *Botrytis cinerea* infection but not developmental senescence, however *anac025-1* appeared to have a delayed dark-induced senescence phenotype in addition to an enhanced susceptibility to Botrytis infection. This may be because dark-induced senescence is a stress condition and not a devel-

opmental stage. Perhaps a timeseries based on dark-induced senescence may reveal a different set of regulatory mechanisms for regulation of *ANAC092* that differ from developmental senescence.

5.3.1.4. Use of transgenic Arabidopsis lines to test network predictions

Transgenic lines of Arabidopsis have been used extensively in this chapter, but they have their own set of limitations. The overexpression of *ANAC056* induced a severely stunted phenotype and a high expression of a number of stress responsive genes. The developmental phenotype induced by overexpression of the transcription factor had caused such a severe response that even if *ANAC092* had been identified as differentially expressed it would have been difficult to prove whether *ANAC092* expression is induced directly by ANAC056 or by stress responses induced by the stunted phenotype induced by *ANAC056* overexpression. An alternative would be an inducible overexpressor construct such as the *ORA59* inducible overexpressor used in Pré *et al.* (2008). This approach enables rapid increase in expression of the gene in response to chemical treatment, which can minimise developmental defects in the early stage of the plant life. However, inducible overexpressors can be 'leaky', causing the gene to be expressed at high background levels. In addition, transient overexpression may cause transcription of some target genes, but may also require additional stress specific factors, so may have a high rate of false negatives.

Therefore, T-DNA lines were used. T-DNA insertion mutants were used to analyse phenotypes and *ANAC092* expression in *MYB108*, *ANAC018*, *ANAC025*, *ANAC056* and *ANAC102* mutants. Of these, both *myb108-1* and *anac025-1* showed a phenotype during dark-induced senescence and *Botrytis cinerea* infection. *ANAC092* expression was diminished in *myb108-1*, but not *anac025-1*, suggesting MYB108 has a greater influence on *ANAC092* expression then ANAC025.

T-DNA lines have a number of limitations though. The validation of a total elimination of functional transcript is very difficult, as there is a certain background rate in all mRNA quantification methodologies. qPCR was used to detect the level of reduction in the three NAC knock-out lines isolated. Only *anac025-1* showed a >90% reduction in gene expression of its target gene. qPCR is a highly sensitive method of mRNA detection and can pick up non-functional transcript generated from transcription of the gene with the insert. Therefore qPCR may pick up minimal expression of non-functioning transcript that is synthesised in *anac018-1* and *anac056-1*. Previously, the presence of mRNA transcript has been observed even though no protein is produced (Monte *et al.*, 2003). To determine whether a knock-out is a complete knock-out, a western blot can be used to detect the presence of protein, as this represents the final outcome of gene expression.

After generating a genome wide insertion library, T-DNA inserts occur at an average rate of 1.5/plant (McElver *et al.*, 2001; Alonso *et al.*, 2003), therefore it is possible

for two or more insertions to occur in an Arabidopsis insertion mutant. These are expected to be 'bred out' as the plant is back-crossed to generate homozygous plants, however there is a likelihood that some remains. Therefore, use of a second T-DNA line would help to validate results more appropriately since the probability of a second mutation occurring and inducing the same mutation is very low. Similarly, lines overexpressing the knock-out gene often complement the results from the knock-out line, by showing the opposite phenotype, or recovering a phenotype when the two lines are crossed. Confirmation of knock-out phenotypes with complementary plant lines such as secondary knock-outs, synthetic miRNA lines or overexpressors would help validate results obtained from a single T-DNA line.

Of the four NAC knock-out lines identified, only *anac025-1* showed a phenotype during *Botrytis cinerea* infection, while *anac025-1* and *anac102-1* had an observable phenotype during dark-induced senescence. *anac018-1* and *anac056-1* appeared to show a wild-type phenotype during dark-induced senescence and *Botrytis cinerea* infection. This could be because *ANAC018* and *ANAC056* are not stress responsive genes, therefore the genes are at low expression levels in the wild type. In the knock-outs, the expression levels are only reduced by a minimal amount and therefore have a very limited affect on phenotype.

It is also possible that *ANAC018* and *ANAC056* are redundant transcription factors, with overlapping functions. They are the closest relatives to each other (Ooka *et al.*, 2003) and share relatively closely related expression profile in all identified expression profiles. *ANAC025* is the next most closely related gene by DNA sequence, but has a differing expression profile in multiple stresses (Ooka *et al.*, 2003). Functional redundancies are common in the highly expanded transcription factors of Arabidopsis (Riechmann, 2002; Danisman *et al.*, 2013; Pérez-Pérez *et al.*, 2013), therefore it is possible that ANAC018 and ANAC056 are functional homologues, which complement each other and recover any phenotype which may be induced by the reduced expression of the other gene in the knock-out.

ANAC092 expression was not significantly expressed in any of the NAC mutants. This may indicate that each NAC is not necessary for *ANAC092* expression, but does not necessarily indicate they do not bind to the *ANAC092* promoter during senescence or Botrytis infection. Therefore, an alternative approach would be detection of DNA binding *in vivo*. True detection of DNA-binding *in vivo* is very difficult. It is possible to extract a transcription factor using Chromatin Immunoprecipitation (ChIP) with a primary antibody, followed by PCR for detection of the target DNA (Fode & Gatz, 2009), however this is restricted by the low availability of primary antibodies for Arabidopsis. In addition, transcription factor proteins are often at a low concentration which makes the process more difficult. Often, overexpressing transcription factors tagged with a GFP, TAP or HA tag offers an alternative (Nakamura *et al.*, 2010; Jensen *et al.*, 2013; Cao *et al.*, 2014), however this is moving away from true *in vivo* conditions and disrupting the natural Arabidopsis cellular environment,

thus removing some of the advantage ChIP had. Similarly, analysing such a large number of transcription factors with tags involves extensive cloning and Arabidopsis transformation, increasing the cost and time required.

An alternative regulatory technique may be more suited to validating the gene regulatory network generated here, but the network does provide a framework describing *ANAC092* expression in two stresses; *Botrytis cinerea* infection and senescence. *ANAC092* is also induced in response to salt stress, oxidative stress, wounding and many more. If an appropriate testing method could be identified, the gene regulatory network could be extended to include a wider variety of stresses and tissue types, generating a full gene regulatory network for *ANAC092*.

6. Use of high-throughput yeast 1-hybrid to determine NAC transcription factor recognition sequences

6.1. Introduction

6.1.1. The NAC domain confers DNA binding capabilities for NAC proteins

The NAC transcription factor family is one of the largest transcription factor families in *Arabidopsis*, comprising some 110 putative or defined NAC genes (Jensen *et al.*, 2010). NAC family transcription factors do not have one specific biological function, instead they mediate a wide range of developmental and stress responsive processes (Olsen *et al.*, 2005b; Nakashima *et al.*, 2012; Puranik *et al.*, 2012). NAC members have been shown to function in meristem formation (Duval *et al.*, 2002), cell wall thickness (Zhong *et al.*, 2006, 2007b), cold stress (Carvallo *et al.*, 2011), salt-tolerance (Nakashima *et al.*, 2012), pathogenic stress (Kim *et al.*, 2012) and many more. As transcription factors with a wide range of function, individual NAC proteins have distinct regulatory targets, that may be similar or different to their relatives.

Unlike their function, NAC protein structure is highly conserved. The titular NAC domain is located at the N-terminus (Ooka *et al.*, 2003) and is responsible for DNA binding and dimerisation (Ernst *et al.*, 2004; Welner *et al.*, 2012). The NAC domain itself does not appear to harbor an intrinsic activation capability (Jensen *et al.*, 2010), instead the transcriptional promotion or repression ability is conferred by the more variable C-terminal domain (Jensen *et al.*, 2010). The high degree of variability in the C-terminal domain allows for different NAC proteins to act as activators or repressors of transcription (Jensen *et al.*, 2010; Hao *et al.*, 2010), generating a greater flexibility for NAC protein action.

6.1.2. Initial identification of the NAC recognition sequence (NAC RS)

If NAC proteins are key to a number of developmental and stress response processes, identification of the regions of DNA recognised by these proteins is critical to understanding their gene regulatory networks. Determination of where and when a NAC is

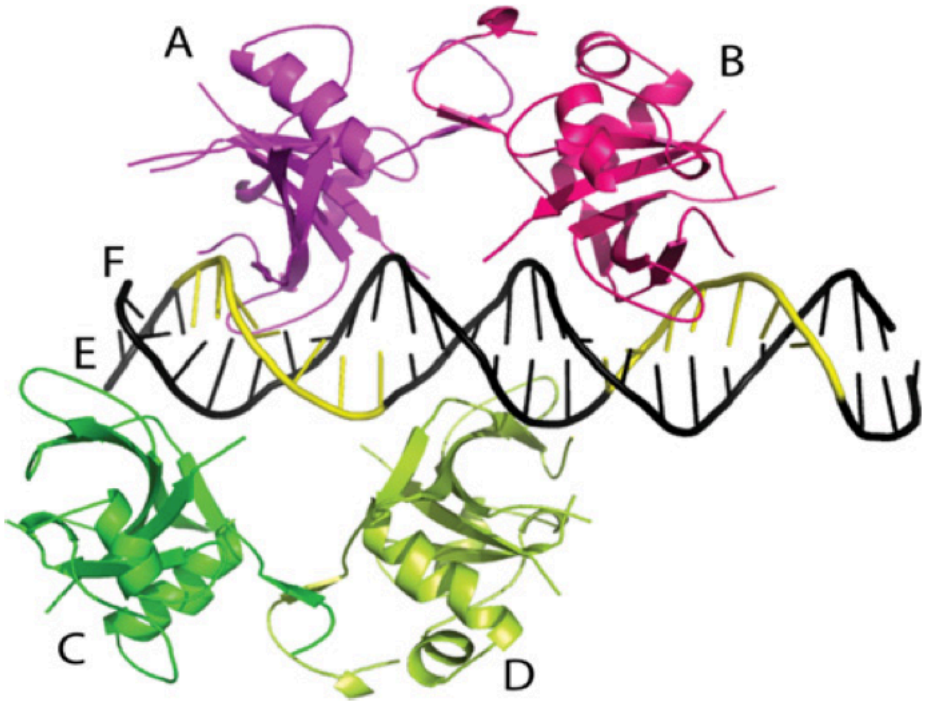


Figure 6.1: **Crystal Structure of ANAC019** 3D structure of ANAC019 bound to DNA as determined by X-ray crystallography. Figure adapted from (Welner *et al.*, 2012)

binding to DNA can help to elucidate many of the downstream mechanisms. Therefore, much work has been dedicated to identifying 'NAC recognition sequences', or NAC-RS.

The initial NAC-RS was identified in an effort to identify transcription factors that regulated drought stress (Tran *et al.*, 2004). In this study, the MYC-like sequence CATGTG from *EARLY RESPONSE TO DEHYDRATION STRESS 1* (*ERD1*) was linked to a *HIS3* and *lacZ* reporter gene and used as bait in a yeast 1-hybrid experiment, where transcription factors were cloned from a drought-stress transcriptome. Three highly related transcription factors were identified, which were later clarified to be ANAC019, ANAC055 and ANAC072. These were shown to recognise the CATGTG sequence in yeast 1-hybrid, EMSA and a protoplast transactivation system (Tran *et al.*, 2004).

Later, an experiment by Olsen *et al.* attempted to define the NAC-RS *de novo*, using no prior knowledge of DNA sequence (Olsen *et al.*, 2005b). To do this, they used a technique known as CASTing (Cyclic Amplification and Selection of Targets) or SELEX (Systematic Evolution of Ligands by Exponential Enrichment). In this technique, two NAC transcription factors (ANAC019 and ANAC092) were purified by heterologous expression in bacteria and incubated with 25bp random oligos. The protein bound to regions of the DNA that contained the recognition site for the NAC proteins, before being purified from the mixture. Unbound oligos were eluted, before

the bound DNA was eluted and amplified using PCR. The PCR product was then used in a second round of CASTing, thus the DNA sequences that bound to the NAC proteins were enriched. This was repeated for a total of six cycles, before the random oligos were sequenced for the enrichment of a DNA sequence recognised by the NAC proteins. Both ANAC019 and ANAC092 appeared to recognise the motif TTnCGT (Olsen *et al.*, 2005a), suggesting this may be the recognition site for these two related NAC proteins.

More recently, the NAC recognition site has been determined for a number of NAC transcription factors including ATAF1 (Jensen *et al.*, 2013), ATAF2 (Wang & Culver, 2012; Huh *et al.*, 2012), ORS1 (Balazadeh *et al.*, 2011), JUB1 (Wu *et al.*, 2012a), NTL6 (Seo *et al.*, 2010) NTL4 (Lee *et al.*, 2012) , ANAC096 (Xu *et al.*, 2013) and CBNAC (Kim *et al.*, 2007a, 2012). ANAC019 has been observed physically forming an interaction with DNA in a crystal structure as a dimer, in a back to back conformation, where each protein bound one of two reverse complement CGTG sequences. In addition, knowledge of the recognition site of ANAC092 has been improved to incorporate a bipartite sequence, composed of two inverted CGT[G/A] sequences separated by 6 base pairs (Jensen *et al.*, 2010). However, *in vivo* it appears that ANAC092 only requires one of these sequences to induce transcription of the *BFN1* gene (Matallana-Ramirez *et al.*, 2013), suggesting ANAC092 may be able to act as a monomer or a dimer.

In all cases, NAC proteins appear to be capable of binding to the core motif CGT[G/A] (Lindemose *et al.*, 2014), suggesting this sequence is recognised by the inherent structure of the NAC domain. The recognition of this core sequence may be specified by a beta-sheet inserted into the major groove of the DNA, which appears to be conserved through all NAC proteins.

Subgroup	Name	ANAC number	ATG	Motif	Reference
I-1	NTL6	ANAC062	AT3G4953	5'-GTAn$_{(9)}$ACGTTTNCTTA-3'	(Seo *et al.*, 2010; Lindemose *et al.*, 2014)
	NTL9/CBNAC		AT4G3558	5'-TAATAATGCTTAG-' TTATAAATTACT -3'	(Kim *et al.*, 2007a, 2012)
I-2	NTL4	ANAC059	AT3G1 5	5'-CGT[A/G]-3'	(Lee *et al.*, 2012)
I-4	NTL8	ANAC004	AT2G273	5'-TTTCCTT-3'	(Lindemose *et al.*, 2014)
II-1	VND7	ANAC003	AT1G7193	5'-Cn$_{(3)}$TTACGT-3'	(Lindemose *et al.*, 2014)
	NST2	ANAC066	AT3G6191	5'-TGCGTnTACG-3'	(Lindemose *et al.*, 2014)
	VND3	ANAC105	AT5G663	5'-TTACGT-3'	(Lindemose *et al.*, 2014)
	SND1	ANAC012	AT1G3277	5'-TTNCGTAA-3'	(Lindemose *et al.*, 2014)
II-3	NAC2/ORE1	ANAC092	AT5G3961	5'-CGTAn$_{(6)}$CGTG-3' 5'-TTnCGT-3'	(Jensen *et al.*, 2010; Matallana-Ramirez *et al.*, 2013; Lindemose *et al.*, 2014)
	ORS1	ANAC059	AT3G1 5	5'-CGTRn$_{(7)}$YACG-3'	(Balazadeh *et al.*, 2011)
III-2	NAP	ANAC029	AT1G6949	5'-CGTA-3' 5'-TTnCGT-3'	(Zhang *et al.*, 2012a; Lindemose *et al.*, 2014)
III-3	ATAF2	ANAC081	AT5G 879	5'-AAATAAGAAGGCAAATATA- ATTTGATAACAACTATT-3'	(Huh *et al.*, 2012)
	ATAF1	ANAC002	AT1G 172	5'-CGTA-3' 5'-AAGTTACGTA-3'	(Jensen *et al.*, 2013; Lindemose *et al.*, 2014)
	ANAC	ANAC019	AT1G5289	5'-CGTG-3' 5'-TTACGT-3'	(Tran *et al.*, 2004; Olsen *et al.*, 2005a) (Bu *et al.*, 2008; Jensen *et al.*, 2010; Welner *et al.*, 2012)
		ANAC055	AT3G155	5'-TTACGT-3'	(Lindemose *et al.*, 2014)
IV-1		ANAC096	AT5G4659	5'-CGTAn$_{(15)}$CGTG-3'	(Xu *et al.*, 2013)
V-1	JUB1	ANAC042	AT2G43	5'-CGTn$_{(7)}$CACG-3'	(Wu *et al.*, 2012b)
VIII-2	VOZ2	VOZ2	AT2G424	5'-CCCGCCC-3'	(Lindemose *et al.*, 2014)

Table 6.1: **Known DNA binding sequences of NAC transcription factors** NAC transcription factors that have had their binding site analysed in one or more experiments are shown alongside their subgroup (defined in Jensen *et al.*, 2010), colloquial name, binding motif and reference for where that binding motif was determined. Underlined is the core CGT[G/A] sequence seen in many NAC recognition sites.

6.1.3. Use of high-throughput protein binding microarrays (PBMs) to analyse NAC:DNA interactions

In many of the sequences identified so far, the NAC recognition sequence appears to require a core CGT[G/A] sequence (Jensen & Skriver, 2014). However, it is not clear how specific NAC transcription factors are recruited. A simple 4 base pair sequence such as CGT[G/A] theoretically occurs 4 times per 1000bp sequence (assuming even distribution of nucleotides), which would be a considerable number of NAC binding sites on the Arabidopsis genome, enough to preclude any specificity of NAC binding. Moreover, phosphatase cleavage experiments imply that the flanking regions on either side of the CGT[G/A] sequence are protected by NAC protein binding, suggesting the DNA sequence immediately adjacent to the binding site are also involved in NAC protein:DNA interactions (Welner *et al.*, 2012). With such a wide range of function for NAC proteins, it is highly likely certain NACs are recruited to specific promoter regions. Therefore, the NAC recognition site for a particular NAC protein must encode an element of specificity not defined by CGT[G/A], to allow particular NAC proteins to be recruited but preclude others.

In an effort to answer this question, Lindemose *et al.* (2014) used a protein binding microarray to analyse the binding affinities of 12 NAC proteins. Protein binding microarrays (PBMs) use a range of random oligos fixed to a glass slide to recruit purified protein to their surface. They allow discovery of *de novo* binding motifs in an unbiased manner, but are limited to DNA sequences of ~10bp (Berger & Bulyk, 2009).

The experiment by Lindemose *et al.* (2014) analysed DNA-binding dynamics of a wide range of NAC family transcription factors, including ANAC019, ANAC055, ATAF1, ANAC092, VND3, VND7, NST2, NTL6, NTL8, SOG1, VOS2 and ANAC002, representing a broad range of biological function and NAC phylogeny. The NAC proteins appeared to bind to three distinct clusters of DNA motifs, which correlated with their location on the phylogenetic tree.

Cluster 1, consisting of ANAC019, ANAC055, ANAC092, ATAF1, NAP, NST2, SND1, VND3 and VND7, bound to the T[G/A]CGT motif identified in Olsen *et al.* (2005a). This cluster could be divided into 2 more clusters, which had further specificity. One cluster, consisting of ANAC092, SND1 and VST2 appeared to bind to TTGCGT, while the rest recognised TACGT.

Conversely, VOZ2 recognised the unique motif CCCGCC, which does not conform to previous NAC recognition sequences and appears to be specific to the VOZ2 gene. VOZ2 contains a zinc-finger N-terminally of the NAC domain, which is crucial for DNA binding (Mitsuda *et al.*, 2004). Therefore VOZ2 may interact with DNA through this structure, rather then its NAC domain. As such, VOZ2 may represent different binding kinetics to other NAC transcription factors.

The final cluster represented the membrane bound NAC transcription factors

NTL6 and NTL8 (Kim *et al.*, 2008; Mi *et al.*, 2012). These membrane bound transcription factors are synthesised in a dormant plasma membrane bound state, before being activated through proteolytic release of their NAC domain. In Lindemose *et al.* (2014) the NAC domains of NTL6 and NTL8 appeared to recognise the 6 base pair motif TT(A/C/G)CTT. This is divergent from previously identified NAC recognition sites, thus indicating different DNA-binding kinetics for membrane bound NAC transcription factors.

The work in Lindemose *et al.* (2014) indicated a number of key features of NAC-DNA binding. Firstly, it appears NAC proteins have recognition for particular DNA sequences encoded in their protein sequence, which in turn means the NAC phylogenetic tree is linked to DNA binding characteristics, i.e., different clades recognise different sequences of DNA. Secondly, while the CGT[G/A] motif is often at the core of NAC recognition sequences, it is by no means universal as certain NAC proteins recognise alternative sequences. In addition, where NAC proteins do recognise CGT[G/A], there is variation that specifies particular groups of NAC proteins.

6.1.4. A yeast 1-hybrid methodology for identification of NAC recognition sequences

While PBMs are capable of unbiased DNA motif detection, they do come with a number of limitations (Berger & Bulyk, 2009). Firstly, the length of oligos fixed to the slide is limited to 10bp. While this can be physically extended to larger sequences, any increase in length exponentially increases the number of possible permutations, e.g., a microarray that uses 12bp oligos requires 16-fold more hybridisation spots then one that uses 10bp oligos. In addition, the short oligos prevent secondary DNA structure believed to be important in protein:DNA interactions. Secondly, PBMs are a fundamentally *in vitro* technique. While considerable effort is made to ensure they reflect the *in vivo* conditions as much as possible (correct pH, salt concentration etc), they still require purified protein, usually from recombinant expression, to function in a monomeric or dimeric fashion with no-cofactors or auxiliary proteins. Finally, the requirement for purified protein limits the number of proteins that could be studied due to the labour and time involved in protein purification.

Yeast 1-hybrid analysis offers an alternative and complementary technique. Compared to PBMs, yeast 1-hybrid is capable of analysing far more transcription factors for DNA binding, but against a smaller number of DNA sequences (Pruneda-Paz *et al.*, 2014). As such, with construction of an appropriate NAC transcription factor library, all NAC proteins could be studied for DNA binding simultaneously. Furthermore, within the PRESTA group, a large number of *Arabidopsis* promoter regions have been cloned by the PRESTA group, generating a resource for real promoters to use as 'bait'. This means any detection of binding will be more applicable to plant tissue then the synthetic DNA sequences used in a PBM. Sequences of 400-500bp

can be analysed in yeast 1-hybrid, which are far longer then the 10bp sequences used in PBMs. Longer sequences are useful because NAC transcription factors have been seen to recognise > 10bp DNA sequences (Wang & Culver, 2012), especially when binding as multimers (Welner *et al.*, 2012). As such, a yeast 1-hybrid promoter region could include auxiliary motifs, dual-binding sites and secondary structures that are important to DNA binding.

Therefore, yeast 1-hybrid offers an alternative method to investigating the binding characteristics of NAC transcription factors. A yeast 1-hybrid library of NAC transcription factors can be constructed from cloned NAC coding regions, which are then analysed for DNA binding against a range of promoter regions. DNA recognition sites for specific NAC proteins could then be inferred from the sequences specifically recognised by individual NAC proteins or clades.

6.1.5. Aim

The aim of this chapter was to develop and use a NAC specific yeast 1-hybrid library for detection of NAC:DNA interactions. From this data, specific DNA binding sequences for particular NAC transcription factors or clades of NAC transcription factors could be predicted from sequence similarities in the recognised promoter regions. Finally, predicted motifs were tested using site-directed mutagenesis.

6.2. Results

6.2.1. NAC-domain based phylogenetic tree

NAC proteins have been shown to recognise DNA through their N-terminal NAC domain (Olsen *et al.*, 2005a; Welner *et al.*, 2012). This highly conserved, unique structure is believed to be the primary method of NAC proteins interacting with DNA, however it has also been shown that the more variable C-terminal region of ANAC092 improves the binding affinity (Lindemose *et al.*, 2014). Nevertheless, it appears the NAC domain is the main component to recognising the NAC recognition sequence. As such, it was decided that the use of a NAC phylogenetic tree based solely on the protein sequence of the NAC domain would be appropriate to separate NAC proteins on the basis of DNA binding characteristics.

The protein sequences of the 110 NAC proteins identified in Jensen *et al.* (2010) were retrieved from TAIR10 annotations using BIOMART (Smedley *et al.*, 2009). The NAC domain for each protein sequence was located and extracted using NCBI conserved domain search (Marchler-Bauer & Bryant, 2004). Where a NAC protein contained two NAC sequences, both NAC domain protein sequences were included as individual entries. In addition, the WRKY domain of WRKY45 was included as an outgroup, since it has low sequence similarity to the NAC domains, but resembles the 3D structure of the NAC domain (Welner *et al.*, 2012).

NAC domains were aligned with Clustal Omega on default 'slow' settings (Sievers *et al.*, 2011), before a phylogenetic tree was constructed using MEGA5 with a James-Thornton-Taylor amino acid substitution model (Jones *et al.*, 1992; Tamura *et al.*, 2011). The phylogenetic tree was visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

The phylogenetic tree was similar to the tree constructed in Jensen *et al.* (2010), although the inclusion of WRKY45 as an outgroup generated a new root and therefore the layout is different. Most NAC proteins were assigned to the same subgroups, with the exception of ANAC001 and ANAC049 which were in clade VII-1 instead of VII-2. ANAC077 moves from clade I-4 to I-2. The clade VI-1, composed of ANAC006 and ANAC097 was split across branches, therefore was disbanded. The branch ANAC006 was previously on was renamed 'VI-4' and included ANAC088 and ANAC027 in addition to ANAC006. ANAC097 was not included in a specific clade and was therefore left as a single member group. In addition, AT3G12910 was included in clade V-1, having not been included as a sequence in Jensen *et al.* (2010).

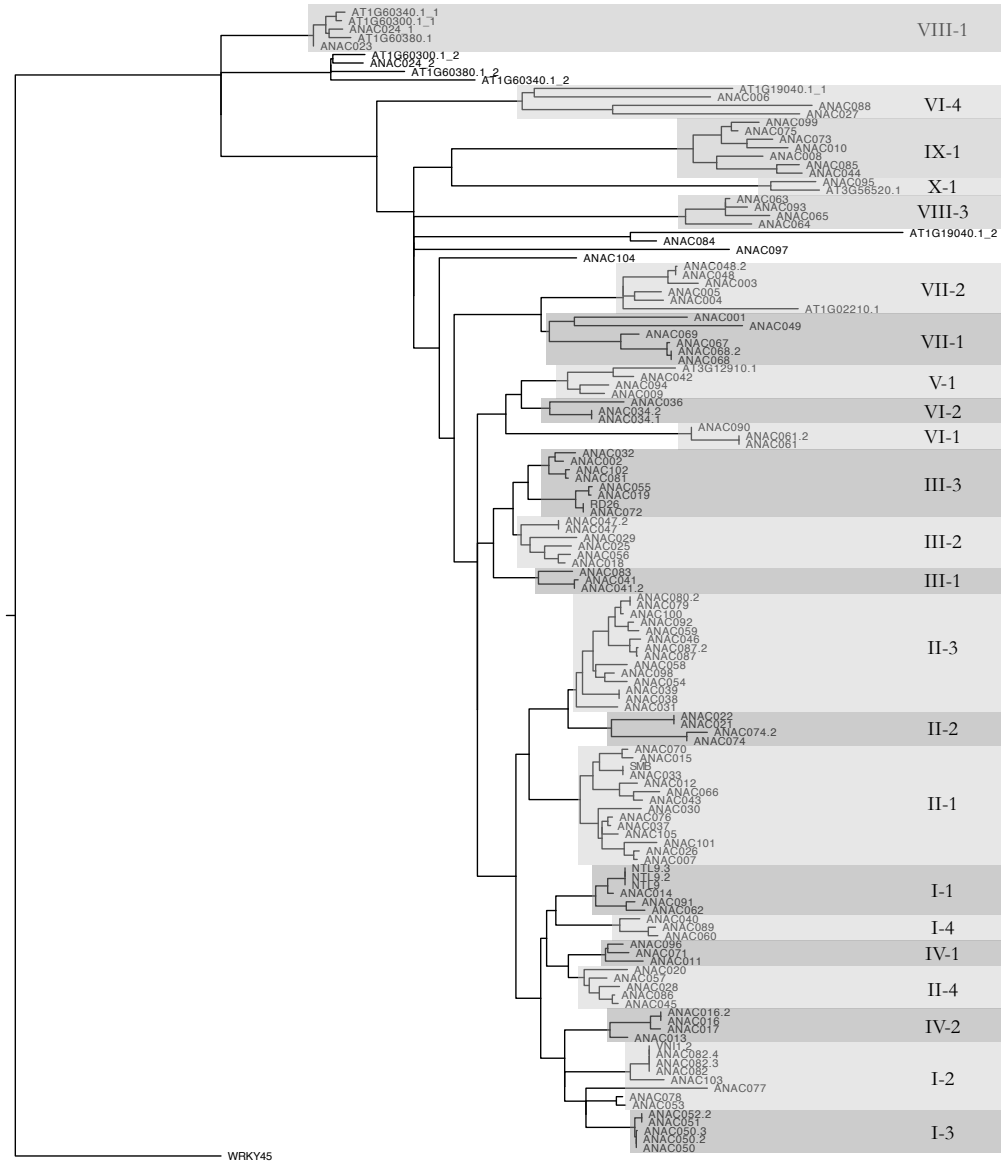


Figure 6.2: **Phylogenetic tree of NAC domains** NAC phylogenetic tree constructed from amino acid sequences of NAC domains, using Clustal Omega for alignment and MEGA5 for construction of phylogenetic tree. All gene models from TAIR10 are included (denoted as .1 or .2). In addition where a NAC gene sequence encoded two NAC domains, both are included as 1 and 2, where 5 represents the sequence closest to the TSS. WRKY45 is included as an outgroup.

6.2.2. NAC specific high-throughput yeast 1-hybrid

6.2.2.1. Generation of a NAC-specific yeast 1-hybrid library

A specific yeast 1-hybrid library of NAC transcription factors was generated and sequence verified. The coding sequence of every NAC transcription factors was cloned either from the PRESTA transcription factor libraries or from Arabidopsis cDNA libraries by Dr. Ana Mendes. In addition, NAC transcription factor encoding a transmembrane domain were cloned without the coding sequence for the transmem-

	1	2	3	4	5	6	7	8	9	1 0	11	12
A	ANAC001	ANAC012	ANAC020	ANAC029	ANAC038/ANAC039	ANAC048	ANAC058	ANAC069	ANAC077	ANAC087	ANAC096	NTL9
B	ANAC002	ANAC013	ANAC022	ANAC030	ANAC040	ANAC050	ANAC059	ANAC070	ANAC078	ANAC088	ANAC097	AT1G02210
C	ANAC003	ANAC014	ANAC023	ANAC031	ANAC042	ANAC051/ANAC052	ANAC060	ANAC071	ANAC079/080	ANAC089	ANAC098	AT1G19040
D	ANAC004	ANAC015	ANAC024	ANAC032	ANAC041	ANAC053	ANAC061	ANAC072	ANAC081	ANAC090	ANAC100	AT1G60300
E	ANAC005	ANAC016	ANAC025	ANAC033	ANAC043	ANAC054	ANAC062	ANAC073	ANAC082	ANAC091	ANAC101	AT1G60380
F	ANAC007	ANAC017	ANAC026	ANAC034/035	ANAC045	ANAC055	ANAC064	ANAC074	ANAC083	ANAC092	ANAC103	AT3G12910
G	ANAC008	ANAC018	ANAC027	ANAC036	ANAC046	ANAC056	ANAC066	ANAC075	ANAC084	ANAC094	ANAC104	GFP
H	ANAC010	ANAC019	ANAC028	ANAC037	ANAC047	ANAC057	ANAC068	ANAC076	ANAC086	ANAC095	ANAC105	Empty

Table 6.2: **Yeast 1-Hybrid library of NAC transcription factors** NAC library used in yeast 1-hybrid, using names derived from TAIR1 annotation, showing arrangement on 96-well plate. Red cells indicate failed transformations and double gene names indicate where a single locus has been annotated as two NAC genes in TAIR10.

brane domain by Mrs. Alison Jackson. This was done because the yeast cellular environment would lack the signal that would trigger translocation from the plasma membrane. As such, removal of the transmembrane domain should prevent them from localising to a membrane.

94 cloned NAC transcription factors were then arranged in a 96-well formation with the addition of GFP and an empty pDEST^{tm}22 vector as negative controls (table 6.2). The 94 transcription factors were chosen based on a number of factors. Where two splice forms of the same transcription factor existed, it was decided to use the longest form, that is, with the fewest excised exons. Many of the shorter splice forms lacked a NAC domain in the alternatively spliced version, therefore they were presumed to lack the appropriate DNA binding domain. In some instances, the coding sequence had been cloned twice, once with the transmembrane domain and once without. In this situation the clone without the transmembrane domain was selected since it would not localise to the membrane. Finally, a small number of NAC clones were not available in time, therefore they were not included. This led to 94 NAC cloned NAC genes available in the pDEST^{tm}22 plasmid.

These NAC transcription factors were cloned into AH109 yeast using the high-throughput protocol described in the methods. It was not possible to transform yeast with clones of ANAC058 and ANAC074. There does not appear to be any conserved feature between these transcription factors, therefore it remains unknown why they were unable to produce viable yeast. In total, the NAC library constructed represents 92 of the 110 NAC proteins indicated in Jensen *et al.* (2010).

6.2.2.2. Selection and screening of Arabidopsis promoter regions for NAC binding

In addition to the promoter fragments cloned in the course of this thesis, a large number of *Arabidopsis* promoter regions have been cloned and screened for DNA binding using yeast 1-hybrid as part of the PRESTA project. Some of these promoter regions had been observed to form an interaction with a NAC transcription factor in this screen, suggesting their sequence contained a recognition motif for a NAC protein. This was used as the preliminary data for the NAC-specific yeast 1-hybrid. Promoter fragments that had previously been observed to produce positive interactions with NAC proteins in yeast 1-hybrid were used as bait in the NAC specific library.

In total, 30 promoter fragments were used for analysis (table 6.3). The 30 promoter fragments came from 26 unique gene loci, with a mixture of distances from the TSS and forward/reverse strand relative to the coding sequence. Promoter fragments were cloned into the pHISLEU2GW vector and sequenced to ensure correct sequence prior to use.

Promoter regions were analysed for NAC binding using yeast 1-hybrid using the same method used previously. Y187 yeast harbouring promoter fragment constructs

Name	Parent gene	Name	Start (relative to TSS)	End (relative to TSS)	Length	Forward/Reverse Strand	Designed by
Y1H6	AT1G27730	STZ	-689	-307	382	Reverse	Stuart McHattie
Y1H7	AT1G27730	STZ	-515	-81	434	Reverse	Stuart McHattie
Y1H8	AT1G27730	STZ	-336	88	424	Reverse	Stuart McHattie
Y1H16	AT2G38470	WRKY33	-918	-477	441	Forward	Laura Bulter
Y1H25	AT3G10820		-348	-1	347	Forward	Claire Hill
Y1H27	AT3G15500	ANAC055	-877	-1	876	Forward	Richard Hickman
Y1H31	AT4G22920	NYE1	122	523	401	Reverse	Stuart McHattie
Y1H37	AT5G39610	ANAC092	-721	-227	494	Reverse	Richard Hickman
Y1H46	AT5G62000	ARF2	-854	-511	343	Forward	Laura Butler
Y1H53	AT3G15500	ANAC055	-668	-382	286	Forward	Richard Hickman
Y1H57	AT1G06160	ORA59	-1074	-646	428	Forward	Peijun Zhang
Y1H73	AT5G05410	DREB2A	-409	-13	396	Forward	Peijun Zhang
Y1H79	AT1G52890	ANAC019	-1021	-708	313	Reverse	Richard Hickman
Y1H139	AT5G50570		-998	-593	405	Reverse	Alex Jironkin
Y1H143	AT5G05090		-714	-286	428	Forward	Alex Jironkin
Y1H147	AT4G31550	WRKY11	-416	-1	415	Reverse	Alex Jironkin
Y1H151	AT3G25760	AOC1	-1000	-587	413	Forward	Alex Jironkin
Y1H157	AT2G44840	ERF13	-995	-590	405	Forward	Alex Jironkin
Y1H159	AT2G44840	ERF13	-416	-1	415	Forward	Alex Jironkin
Y1H167	AT1G09030	NF-YB4	-713	-289	424	Reverse	Alex Jironkin
Y1H215	AT3G01420	DOX1	-1024	-607	417	Reverse	Peijun Zhang
Y1H235	AT4G11910		-400	-1	399	Forward	Peijun Zhang
Y1H325	AT3G28920	AtHB34	-712	-308	404	Reverse	Peijun Zhang
Y1H381	AT5G12840	NF-YA1	-427	-1	426	Reverse	Peijun Zhang
Y1H387	AT1G30500	NF-YA7	-402	-1	401	Reverse	Peijun Zhang
Y1H395	AT2G34720	NF-YA4	-423	-1	422	Reverse	Peijun Zhang
Y1H496	AT3G26830	PAD3	-414	-1	413	Forward	Peijun Zhang
Y1H499	AT5G13180	ANAC083	-410	-1	409	Forward	Peijun Zhang
Y1H507	AT2G33710		-1000	-600	400	Reverse	Jo Rhodes
Y1H508	AT3G53600		-400	-1	399	Forward	Jo Rhodes

Table 6.3: **Promoter fragments screened in yeast 1-hybrid against a NAC specific transcription factor library** Table shows the promoter region analysed for NAC protein binding in yeast 1-hybrid. Included is the locus and name of the gene for each promoter region, in addition to the location from the TSS and the strand of DNA on the Arabidopsis genome. Promoter fragments were designed by various members of the PRESTA group, this information is included. Y1H37 corresponds to fragment 2, from chapter 4 of this thesis.

were mated with AH109 yeast harbouring transcription factor constructs on solid media, before being transferred to positive control (SD minus Leu and Trp) and selective media (SD minus Leu, Trp and His) with multiple concentrations of 3-aminotriazole (3-AT), which was used as an antagonist of HIS3 activity. Empty pDEST^{tm}22 and pDEST^{tm}22-GFP colonies were used to normalise the levels of 3-AT required. Different fragments required different concentrations of 3-AT to reduce background levels of HIS3 activity.

6.2.2.3. Patterns of NAC binding in yeast 1-hybrid analysis

Of the 92 NAC proteins screened for DNA binding in yeast 1-hybrid, 35 interacted with one or more regions of DNA (table 6.4). This represents only 1/3 of the total NAC proteins present in the yeast 1-hybrid library. It is possible that this third are the only NAC transcription factors capable of acting in yeast 1-hybrid, as the others may fold incorrectly in yeast or require heterodimerisation or post-translational modification. Alternatively, they may be specific for other promoter regions that were not screened in this experiment.

Of the transcription factors that did interact with DNA in yeast 1-hybrid, there is a relatively even spread of interactions, with no promiscuous NAC protein that can bind to an abnormal number of sequences. ANAC038/039 appeared to interact with the highest number of promoter regions, with eight independent interactions. The next greatest number of interactions is ANAC089, with seven. The number of interactions then decreases in a fairly linear manner to zero. This stable progression of number of interactions by NAC proteins suggested that there is no generic 'DNA-binding' structure in certain NAC proteins, which can bind to DNA with little or no sequence specificity. That is, all NAC proteins that are effective in yeast 1-hybrid are particular about recognising a certain sequence. Therefore any binding capabilities should be conferred by the combination of the NAC protein structure and promoter sequence.

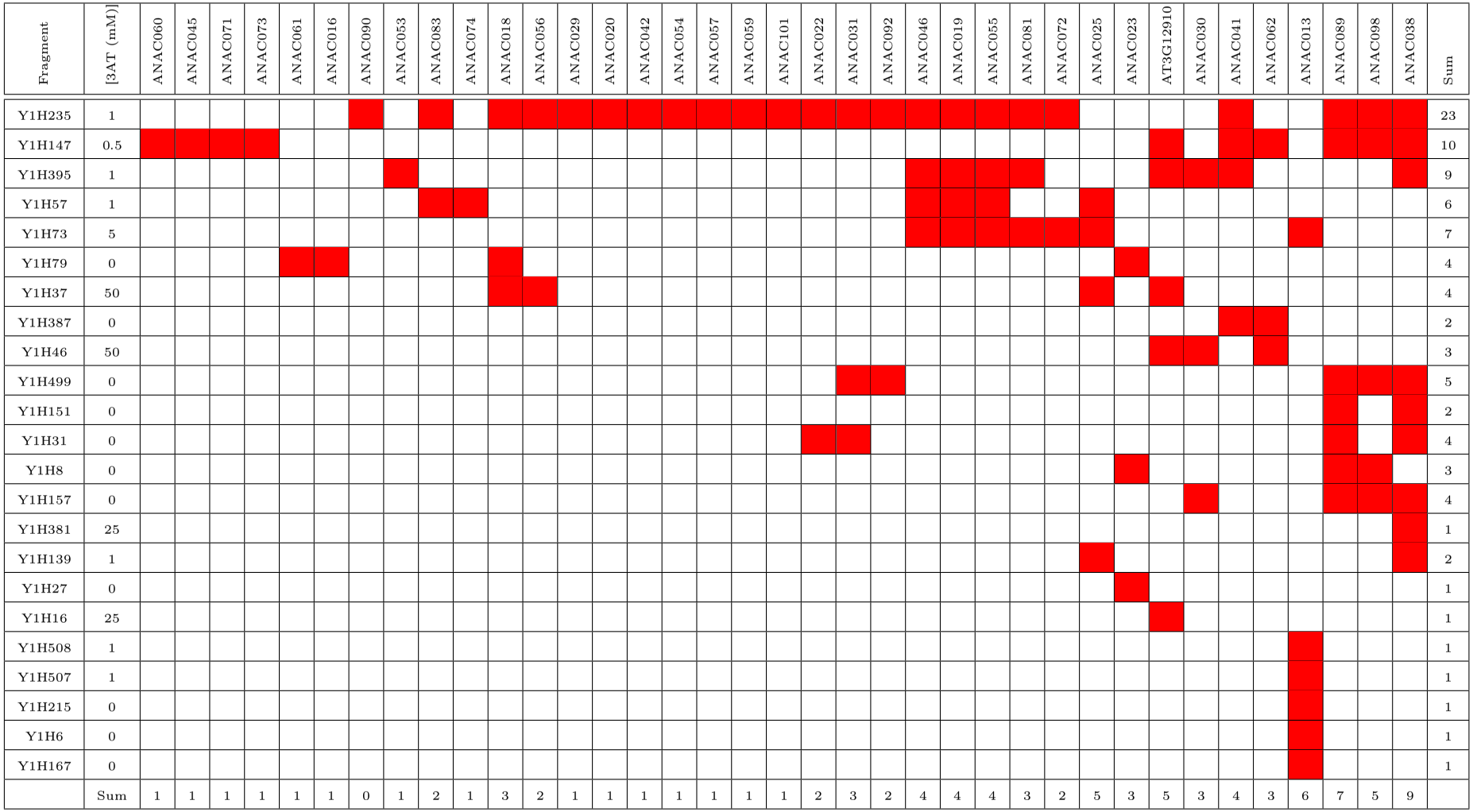
There did not appear to be a particular clade of NAC family transcription factors that demonstrated a higher propensity to DNA binding, suggesting the ability to function in DNA is not an inherent property of particular groups of NAC proteins. Subgroup VII (1 & 2) did not show a single instance of binding to DNA, perhaps indicating this subgroup do not function in yeast 1-hybrid, but as one of the smallest subgroups of NAC proteins it is also possible this result is by chance.

6.2.2.4. Patterns of NAC binding in yeast 1-hybrid by promoter region

Promoter regions demonstrated varying binding characteristics (table 6.5). In spite of originally binding to a NAC protein in a yeast 1-hybrid library screen that was used to form the initial dataset, seven promoter regions did not appear to recruit a single NAC protein. Eight promoter regions recruited a single NAC protein, while

Name	ATG	Subgroup	Total
ANAC038/039	AT2G24430	II-3	8
ANAC089	AT5G22290	I-4	7
ANAC098	AT5G53950	II-3	5
Uncharacterised NAC	AT3G12910	V-1	5
ANAC013	AT1G32870	IV-2	4
ANAC019	AT1G52890	III-3	4
ANAC046	AT3G04060	II-3	4
ANAC055	AT3G15500	III-3	4
ANAC018	AT1G52880	III-2	3
ANAC023	AT1G60280	VIII-1	3
ANAC025	AT1G61110	III-2	3
ANAC030	AT1G71930	II-1	3
ANAC031	AT1G76420	II-3	3
ANAC041	AT2G33480	III-1	3
ANAC062	AT3G49530	I-1	3
ANAC081	AT5G08790	III-3	3
ANAC022	AT1G56010	II-2	2
ANAC056	AT3G15510	III-2	2
ANAC072	AT4G27410	III-3	2
ANAC083	AT5G13180	III-1	2
ANAC092	AT5G39610	II-3	2
ANAC016	AT1G34180	IV-2	1
ANAC020	AT1G54330	II-4	1
ANAC029	AT1G69490	III-2	1
ANAC042	AT2G43000	V-1	1
ANAC045	AT3G03200	II-4	1
ANAC053	AT3G10500	I-2	1
ANAC054	AT3G15170	II-3	1
ANAC059	AT3G29035	II-3	1
ANAC060	AT3G44290	I-4	1
ANAC061	AT3G44350	VI-3	1
ANAC071	AT4G17980	IV-1	1
ANAC073	AT4G28500	IX-1	1
ANAC074	AT4G28530	II-2	1
ANAC101	AT5G62380	II-1	1

Table 6.4: **Summary of NAC:DNA interactions by NAC protein** List of NAC proteins that formed successful interactions with DNA in yeast 1-hybrid against a one or more promoter regions, with the number of promoter regions.



Fragment	[3AT (mM)]	ANAC060	ANAC045	ANAC071	ANAC073	ANAC061	ANAC016	ANAC090	ANAC053	ANAC083	ANAC074	ANAC018	ANAC056	ANAC029	ANAC020	ANAC042	ANAC054	ANAC057	ANAC059	ANAC101	ANAC022	ANAC031	ANAC092	ANAC046	ANAC019	ANAC055	ANAC081	ANAC072	ANAC025	ANAC023	AT3G12910	ANAC030	ANAC041	ANAC062	ANAC013	ANAC089	ANAC098	ANAC038	Sum
Y1H235	1							■		■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					■			■	■	■	23
Y1H147	0.5	■	■	■	■																										■		■	■		■	■	■	10
Y1H395	1								■															■	■	■	■				■	■	■					■	9
Y1H57	1									■	■													■	■	■			■										6
Y1H73	5																							■	■	■	■	■	■					■				7
Y1H79	0					■	■					■																		■								4
Y1H37	50											■	■																■		■							4
Y1H387	0																															■	■					2
Y1H46	50																														■	■		■				3
Y1H499	0																					■	■												■	■	■	5
Y1H151	0																																		■		■	2
Y1H31	0																				■	■													■		■	4
Y1H8	0																													■					■	■		3
Y1H157	0																															■			■	■	■	4
Y1H381	25																																				■	1
Y1H139	1																												■								■	2
Y1H27	0																													■								1
Y1H16	25																														■							1
Y1H508	1																																	■				1
Y1H507	1																																	■				1
Y1H215	0																																	■				1
Y1H6	0																																	■				1
Y1H167	0																																	■				1
	Sum	1	1	1	1	1	1	0	1	2	1	3	2	1	1	1	1	1	1	1	2	3	2	4	4	4	3	2	5	3	5	3	4	3	6	7	5	9	

Table 6.5: **NAC-specific yeast 1-hybrid results** Yeast 1-hybrid results for each promoter region, showing concentration of 3-AT to suppress background activity of HIS3, as well as positive interactions by NAC proteins on that promoter and total number of interacting NAC proteins.

three recruited just two NAC proteins. Eleven promoter regions recruited more then two NAC family transcription factors. Fewer promoter regions recruited more NAC proteins, i.e., there was an inverse correlation between the number of promoter regions and number of NAC transcription factors that bound to them. However, one particular promoter region, Y1H-235, exhibited extraordinary NAC binding characteristics in yeast 1-hybrid.

6.2.2.5. A region of the *STAY-GREEN 2* promoter appears to show enhanced ability to recruit to NAC proteins in yeast 1-hybrid

The promoter fragment Y1H-235 formed a positive interaction with 23 NAC transcription factors, representing over 25% of all observed interactions in this experiment. Y1H-235 encodes the first 400bp upstream from the transcription start site of *STAYGREEN 2* (*SGR2*), a protein which is involved in repression of leaf senescence (Sakuraba *et al.*, 2014). SGR2 appears to bind to subunits of the LHCII complex at the thylakoid membrane (Sakuraba *et al.*, 2014). Expression of *SGR2* increases as the leaf senesces, suggesting a positive role in leaf senescence, but overexpressors of *SGR2* exhibit delayed leaf senescence while mutant plants deficient in *SGR2* expression show accelerated senescence (Sakuraba *et al.*, 2014). Since SGR2 expression increases during leaf senescence but appears to retard the process, it has been suggested to act as a negative feedback mechanism ensuring chlorophyll degradation does not accelerate in an uncontrolled manner. Interestingly, SGR2 interacts with the same set of proteins to the close relative SGR1, but with a lower affinity (Sakuraba *et al.*, 2012, 2014). *SGR1* mutants have the opposite phenotypes to *SGR2* mutant plants, suggesting an antagonistic mechanism between the two proteins as they bind to photosynthetic machinery (Sakuraba *et al.*, 2012, 2014).

In the yeast 1-hybrid screen, 23 NAC transcription factors bound to the promoter region of *SGR2*, suggesting it encodes a DNA sequence that has a high affinity for NAC transcription factor binding. In particular, NAC transcription factors from subclade II, III and V appeared to bind to Y1H-235, while NACs from subclade I and VIII did not. It is likely that the promoter region of SGR2 encodes a DNA sequence that is responsible for recruiting many NAC proteins from many different clades. Perhaps the promoter region contains a core recognition sequence that can be recognised by many NAC proteins, but lacks specificity that prohibits other proteins from binding and therefore the *SGR2* promoter is highly promiscuous for NAC binding. It is unclear why this is, although it appears many of the NAC transcription factors that bound to Y1H-235 were upregulated during the course of senescence (figure 6.4). Therefore, the promoter region of *SGR2* may encode a recognition sequence that allows senescence-related NAC transcription factors to bind. The transcription promoted by these proteins may be a key method of increasing levels of *SGR2* transcript during senescence (Sakuraba *et al.*, 2014). However, more work is needed to

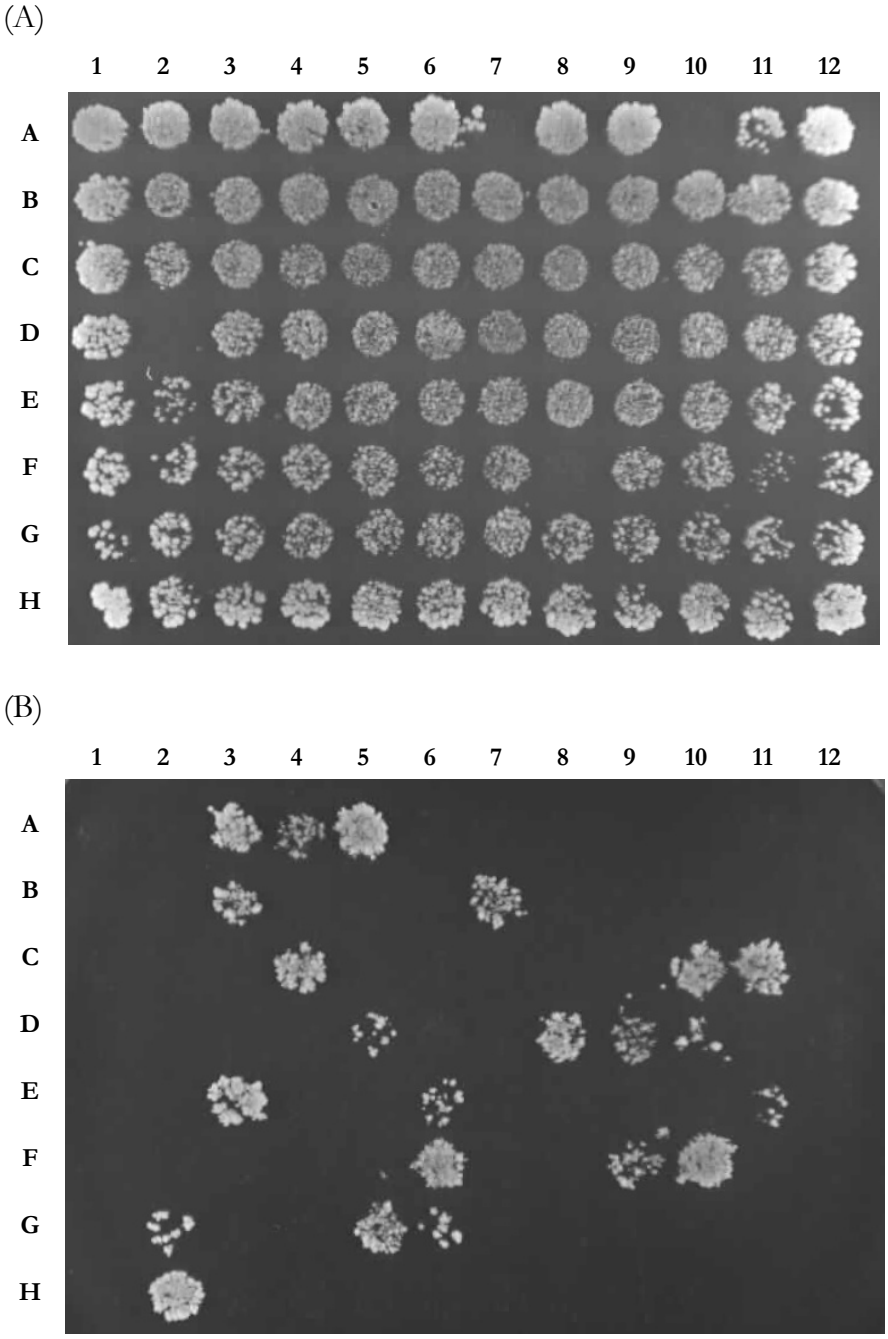


Figure 6.3: **NAC specific yeast 1-hybrid of *STAYGREEN 2* promoter region** Yeast 1-hybrid of Y1H-235 against the NAC specific transcription factor library. Y1H-235 encodes the 4 bp region upstream from the *STAYGREEN 2* transcription start site. (A) Growth on SD minus Leucine and Tryptophan media. This is selective for correct mating, therefore any colonies are diploid yeast containing pHISLEU2GW-Y1H-235 and pDEST^{tm}22-*NAC*. (B) Growth on SD minus Leucine, Tryptophan and Histidine, which is selective for *HIS3* expression from Y1H-235, therefore colonies represent a positive interaction on the promoter.

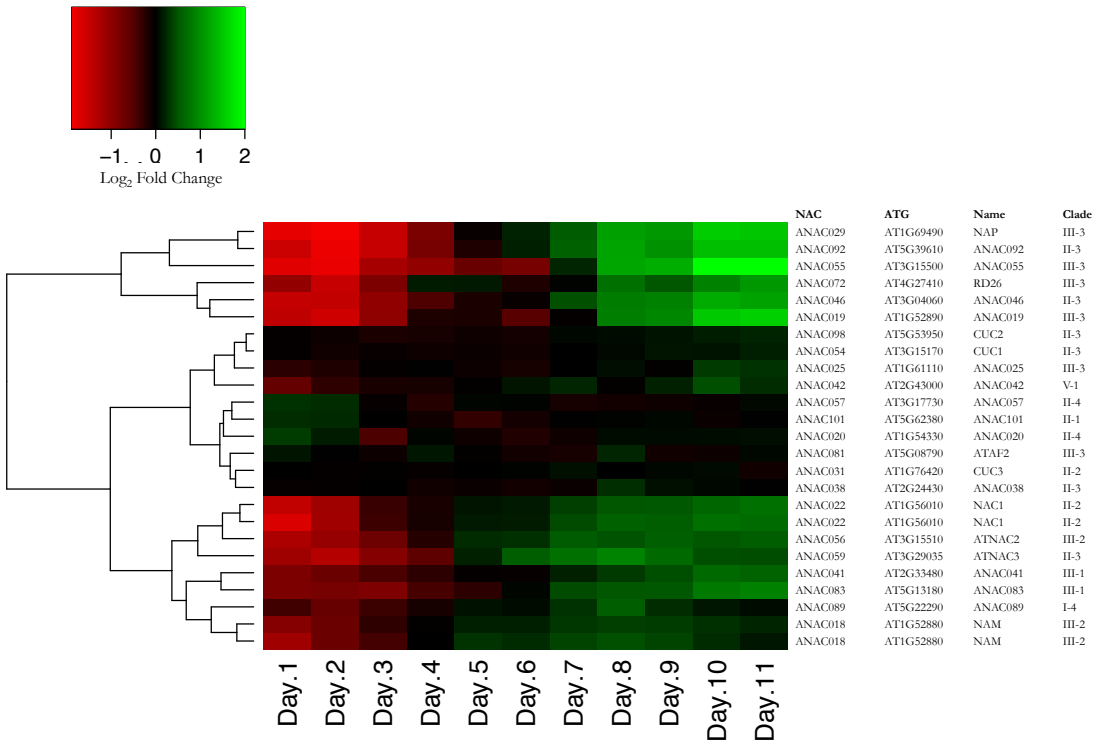


Figure 6.4: **Heatmap of NAC transcription factors that bind to Y1H-235 in yeast-1 hybrid** NAC transcription factors that bound to the promoter region Y1H-235, which encoded the 4 bp immediately upstream from the TSS of *SGR2*. Shown are the expression levels of these transcription factors during age-induced leaf senescence as determined by the PRESTA timeseries microarrays (Breeze *et al.*, 2011).

confirm this.

6.2.3. Determination of novel NAC recognition sequences

6.2.3.1. Analysis of DNA sequences in promoter regions that successfully formed an interaction with a NAC protein

The aim of this chapter was to determine specific NAC binding motifs using yeast 1-hybrid. With knowledge of the transcription factors that could bind to particular promoter regions, the DNA binding characteristics of one or more NAC transcription factors could be inferred from the promoter sequences. Initially, it was thought that a core recognition sequence for all NAC proteins might be detected from the cumulative data generated by the yeast 1-hybrid. All promoter fragments that recruited one or more NAC family transcription factors were analysed for conserved motifs using the

MEME software (Bailey & Elkan, 1994; Bailey *et al.*, 2009). Motif detection software such as MEME has a tendency to produce long motifs with low specificity rather than short highly conserved motifs, as these are more statistically valid, though they are less biologically plausible (Stewart *et al.*, 2012). As such a number of MEME parameters were altered from the default settings. Firstly, since a NAC recognition motif was presumed to occur in all sequences, MEME was set to detect a single instance of the motif every DNA sequence. Secondly, the motif size was reduced from the default 6-50 bp to 4-12bp as this is more likely to represent a DNA recognition sequence for a NAC protein. Finally, all promoter sequences screened against the NAC library that did not bind to a single NAC protein in yeast 1-hybrid were included as a negative control, to reduce false-positive results.

All promoter regions that bound to one or more NAC proteins were analysed using MEME with these parameters. A number of motifs were detected, however they all appeared to consist of poorly conserved highly variable DNA sequences (figure 6.5). Most were characterised by two or three conserved nucleotides in a 10 bp motif, with little conservation of DNA sequence outside of these residues. This is indicative of motifs that are overfitted to poorly-conserved sequences or noisy data, therefore it seems likely that these motifs are not true NAC recognition sequences. However, there is a high occurrence of the 2bp sequence CT. It is plausible that this sequence may facilitate NAC binding, although the mechanism behind this is unclear.

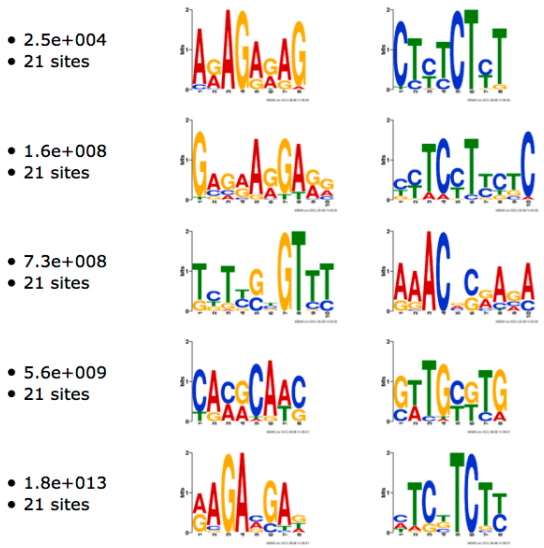


Figure 6.5: **MEME analysis of all promoters that formed positive interactions with NAC proteins in yeast 1-hybrid** Motifs that were produced by MEME analysis when analysing all promoter regions that produced a positive result with any NAC protein in the NAC specific yeast 1-hybrid. Top five motifs are shown, as ranked by the e-value (a measure of statistical significance used by MEME, which essentially compares the log likelihood of a motif of the same width and base composition appearing in a set of random sequences).

6.2.3.2. Analysis of DNA sequences in promoter regions that successfully formed an interaction with a NAC protein of specific clades

Previously, it has been shown that different subgroups of NAC proteins recognise different DNA sequences (Jensen *et al.*, 2010). Therefore, it is possible that different subsets of the 35 NAC proteins tested before may recognise distinct DNA sequences, that are dissimilar to the recognition sequence of other NAC proteins. In this way, particular motifs could recruit certain subclades of NAC proteins.

To analyse the binding characteristics of certain subgroups of NAC proteins, the yeast 1-hybrid results from individual subgroups were analysed together. For example, any promoter region that recruited a subgroup I NAC transcription factor was analysed for conserved sequences in MEME. This is because, in theory, all of those promoter regions could recruit a subgroup I NAC protein, therefore they must share a DNA sequence that subgroup I NAC proteins can recognise. MEME was used with the same parameters as before, but using the new inputs. The results are summarised in table 6.6.

Subgroup I Four NAC transcription factors from subgroup I bound to a total of seven promoter regions in yeast 1-hybrid. MEME analysis of these promoter fragments appeared to suggest conservation of an eight-mer largely composed of C and T residues. The full motif is 5'-C[C/T]CT[C/G]TT[T/G]-3', which includes five residues conserved across all promoters, with some variation in the other nucleotides. Comparison to known plant motifs in TOMTOM (JASPAR core 2014 database, Gupta *et al.* 2007; Mathelier *et al.* 2014) reveals no significant similarity to known transcription factor binding sites. This may represent a novel transcription factor recognition motif for subgroup I NAC transcription factors.

Subgroup II Subgroup II is the largest group of NAC transcription factors. In line with this, more transcription factors from subgroup II produced a positive result then any other subgroup. Twelve promoter regions were recognised by a member of NAC subgroup II. MEME analysis of these promoters revealed a conserved C and T rich motif, similar to the promoters of subgroup I. The full motif is 5'-CTCTCNNNTT-3', which also is similar to the subgroup I motif previously detailed, although this is unsurprising considering all but one of the promoters included in subgroup I analysis were also included in the analysis for subgroup II. This may indicate subgroup I and II NAC transcription factors recognise similar sequences.

Interestingly, both the motif identified for subgroup I and II retained a double thymine sequence. This has previously been seen to be in the recognition motif of ANAC092, SND1 and VST2 (Zhong *et al.*, 2007a; Matallana-Ramirez *et al.*, 2013; Lindemose *et al.*, 2014), therefore it is possible a double TT sequence may be critical for DNA-binding across many NAC proteins. Perhaps the NAC domain protein structure inherently recognises a TT sequence.

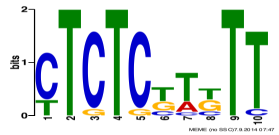
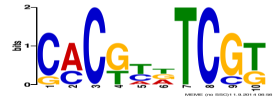
Subgroup	NAC proteins	Promoter regions	Highest Scoring Motif
Subgroup I	ANAC062 ANAC059 ANAC089 ANAC060	Y1H_46 Y1H_31 Y1H_395 Y1H_151 Y1H_8 Y1H_387 Y1H_147	
Subgroup II	ANAC030 ANAC046 ANAC101 ANAC031 ANAC021/022 ANAC092 ANAC074 ANAC059 ANAC038/039 ANAC054 ANAC098 ANAC020 ANAC045	Y1H_46 Y1H_499 Y1H_235 Y1H_147 Y1H_31 Y1H_395 Y1H_57 Y1H_157 Y1H_151 Y1H_381 Y1H_8 Y1H_73	
Subgroup III	ANAC041 ANAC029 ANAC083 ANAC019 ANAC025 ANAC055 ANAC018 ANAC081 ANAC056 ANAC072	Y1H_395 Y1H_387 Y1H_57 Y1H_37 Y1H_79 Y1H_147 Y1H_235 Y1H_73	
Subgroup V	AT3G12910 ANAC042	Y1H_37 Y1H_46 Y1H_235 Y1H_147 Y1H_16 Y1H_395	
Subgroup IV	ANAC013 ANAC016 ANAC071	Y1H_6 Y1H_508 Y1H_42 Y1H_147 Y1H_507 Y1H_79	
Subgroup VIII	ANAC023	Y1H_79 Y1H_27 Y1H_8	

Table 6.6: **MEME results from individual clades of NAC proteins** Table showing subgroup of NAC protein as determined by Olsen *et al.* 2005a, Jensen *et al.* 2010 and this work (figure 6.2). 'NAC proteins' column shows any NAC transcription factor from that family which bound to one or more promoter regions in yeast 1-hybrid. 'Promoter regions' column shows any promoter region which was bound by a NAC protein from that particular subgroup. These promoter regions were analysed using MEME for any conserved DNA sequences. The highest-scoring motif, as determined by MEME e-value, is illustrated in the final column.

Subgroup III Subgroup III includes ANAC018, ANA025 and ANAC056, the transcription factors previously observed to bind to the promoter region of *ANAC092.* Ten members of this subgroup bound to one or more of eight promoter regions in yeast 1-hybrid, including the promoter region from *ANAC092* (Y1H-37). MEME analysis of these eight promoter regions indicated a highly conserved seven base pair motif. The DNA sequence of this motif was 5'-AGGTTAC-3' and was almost identical in all of the promoter fragments (figure 6.6) . This motif has a complex sequence, composed of purines and pyrimidines as opposed to a simple A/T rich region present in many non-coding sequences. Analysis with FIMO revealed the sequence AGGTTAC is not present in the other promoter fragments screened in this experiment, supporting the hypothesis that this is a specific NAC subgroup III protein. Comparison to known motifs in the JASPAR core 2014 database using TOMTOM (Gupta *et al.*, 2007) revealed no significant similarity to a known motif, although the central portion resembles the MYB77 recognition motif sequence, C[G/A]GTT (Romero *et al.*, 1998).

Subgroup IV The NTL proteins are membrane bound NAC proteins NAC proteins which translocate to the nucleus after activation (Kim *et al.*, 2007b). Three of these transcription factors, *NTL1/ANAC013*, *NTL3/ANAC016* and *NTL7/ANAC017* are grouped in the subclade IV NAC transcription factors. These NACs bound to 6 promoter regions, which were analysed for conserved motif sequences. Interestingly, a bipartite motif was detected, showing two small 4bp motifs separated by a 3bp region. The sequence of the motif is therefore 5'-CACnnnTCGT-3'. This double motif characteristic has been observed for ANAC092:DNA interactions in EMSA (Jensen *et al.*, 2010) and ANAC019:DNA interactions in a crystal structure (Welner *et al.*, 2012), however the specific sequence identified here has not been seen before. In the previous examples, the NAC protein has bound to the DNA as a dimer, with one protein binding to each DNA site. The non-coding sequence between the two recognition sites has previously been seen to be longer, roughly 6 base pairs for ANAC092. Nevertheless, this double motif could represent a binding site for ANAC013, ANAC016 and ANAC017 dimers to bind to DNA.

Subgroup VIII The only NAC transcription factor from subgroup VIII that bound to any DNA in yeast 1-hybrid was ANAC023, which has not been the focus of a specific study so far, but has been seen to be increased in expression in response to ABA treatment (Mishra *et al.*, 2014). ANAC023 bound to three promoter regions, which were analysed for conserved motifs in MEME. A 5'-CCAC[G/T]TGTC-3' motif was identified as highly conserved between the three promoters. This was alongside a number of other highly conserved motifs. There was around a 50% sequence conservation between all three promoters, suggesting they share common origins. Indeed, Y1H-79 and Y1H-27 are upstream on the genome of ANAC019 and ANAC055, re-

spectively. These two proteins are very similar to each other and function together in jasmonate and ABA signalling (Bu *et al.*, 2008; Jiang *et al.*, 2009) and senescence (Hickman *et al.*, 2013). Y1H-8 is upstream on the genome of *SALT TOLERANCE ZINC FINGER* (*STZ*) or *ARABIDOPSIS ZINC FINGER 1* (*ZAT1*), an unrelated transcription factor, but it shares much of its promoter region with *ANAC019* and *ANAC055*. Therefore there may be some conservation in promoter regions that is not present in coding sequences, which causes the expression of the three genes to be coregulated. This means that ANAC023 may regulate ANAC019, ANAC055 and STZ simultaneously. However, it is difficult to determine where ANAC023 binds on this promoter, since there are many instances of homology on the promoter region.

6.2.3.3. Validation of the putative NAC-III motif using yeast 1-hybrid

Since the motif on promoter regions that bound to subgroup III NAC transcription factors was highly conserved, novel and complex, it was decided to investigate this motif further. The motif is present on the *ANAC092* promoter region, therefore it may be responsible for the NAC transcription factor binding to the *ANAC092* promoter previously observed in this thesis. The motif was named the NAC-III recognition motif.

MEME was set to compare DNA sequences based on a single instance of each motif. These settings were used to ensure that MEME identified a motif in every sequence (any other setting allows for zero motifs per sequence). To determine if the motif occurred more then once in a promoter sequence, FIMO (Grant *et al.*, 2011) was used with the MEME motif matrix for the eight promoter regions recognised by NAC proteins. For seven of the promoter regions, the motif only appeared once, suggesting only one motif is required for NAC transcription factor binding (figure 6.6).

There were two instances of the motif in Y1H-235, but with a slight deviation, namely an A to C at the start of the motif and a C to T at the end, creating a CGGTTAT sequence. If the NAC-III recognition motif is required for subgroup III specific NAC binding, then perhaps the slight deviation from the NAC-III motif on Y1H-235 allows different NAC transcription factors to bind, which may cause the extraordinary NAC binding characteristics observed with this fragment (figure 6.3). Alternatively, the presence of two motifs in Y1H-235 may enhance NAC transcription factor binding, therefore causing the larger number of NAC binding instances observed for this fragment.

The hypothesis that was tested is that the NAC-III motif is required for recognition of subgroup III NAC proteins, but that the deviation and duplication of the NAC-III motif in fragment Y1H-235 allows multiple NAC proteins to bind, causing the extraordinary results seen in table 6.4. To test this, the NAC-III motif in three promoter fragments was mutated using site-directed mutagenesis. Commonly

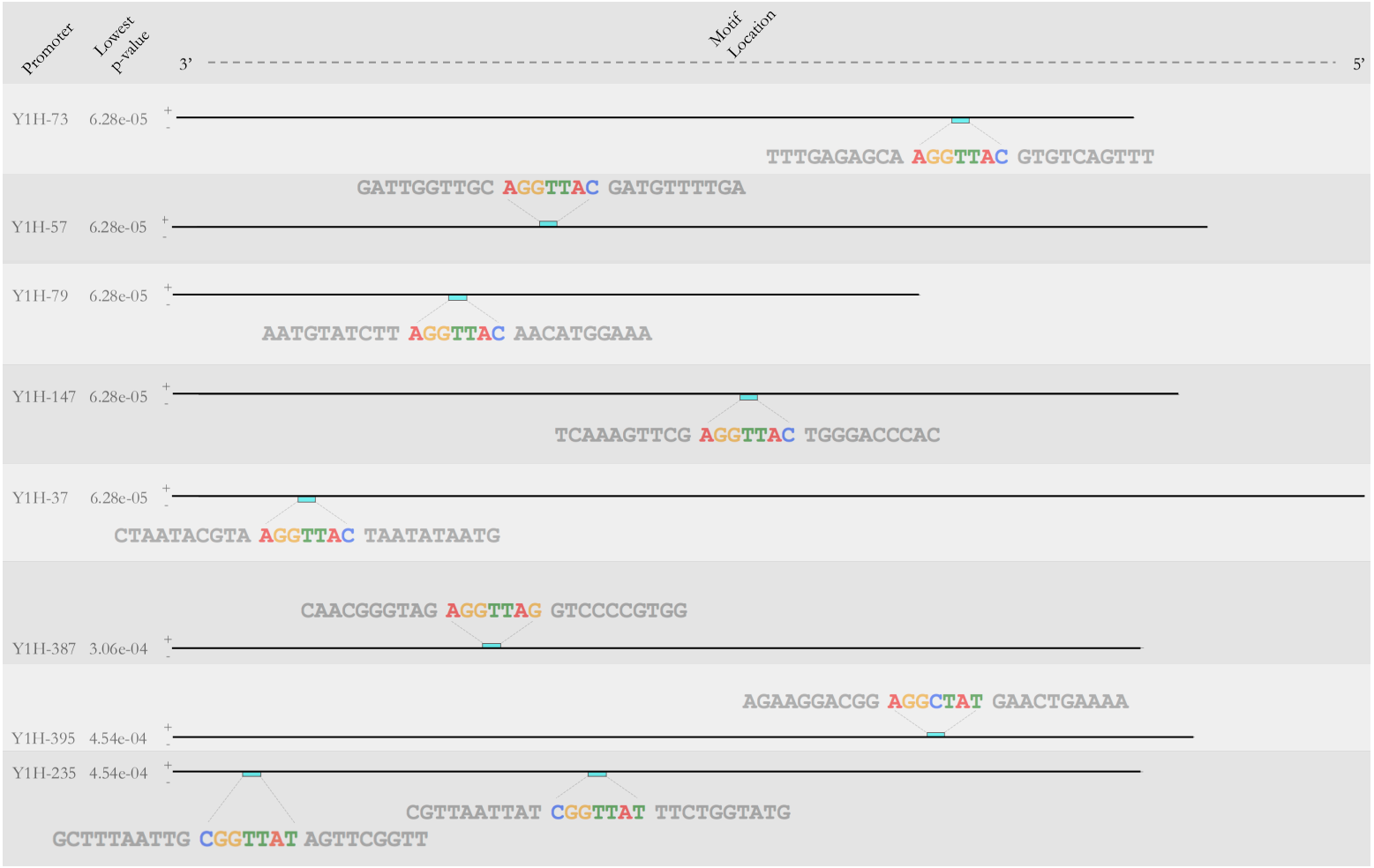


Figure 6.6: **Location of putative NAC-III Recognition sites on promoter fragments** Location of putative NAC-III binding motif, as identified by MEME and FIMO. Promoter fragment is shown with p-value determined by MEME for fragment, with accompanying diagram of promoter fragment displaying 3' - 5' direction (i.e. coding region is to the left). Location of motif and localised sequence is shown by expanded section. Above the diagram indicates positive strand, below the diagram indicates negative strand.

in experiments involving mutated DNA motifs, the motif is converted to $(A/T)_n$, however, there was concern that altering a seven base pair motif with a number of C/G residues may cause altered secondary structure that affected results, so instead the NAC-III motif was converted from 5'-AGGTTAC-3' to 5'-TTTCCCC-3'. The promoter regions Y1H-147, Y1H-37 and Y1H-235 were mutated to eliminate the putative NAC-III motif. Y1H-37 was mutated because it encoded a sequence from the promoter of *ANAC092* and was therefore relevant to the rest of this thesis. Y1H-235 was also mutated in an attempt to understand the unusual NAC binding characteristics observed for this fragment. Because this promoter region had two instances of the motif, each motif was mutated as an individual mutant before being used to create a double mutant with both motifs eliminated.

The wild-type and mutated promoter regions were tested simultaneously for NAC binding in the yeast 1-hybrid system. Disappointingly, there was very little difference between the wild type and mutated promoter region for any of the promoter regions (figure 6.7, 6.8 and 6.9). A small difference was observed for Y1H-235, where ANAC101/VND1 did not interact with Y1H-235A, Y1H-235B or Y1H-235AB but did interact with Y1H-235. Conversely, ANAC090 bound to the mutated Y1H-235, but not the wild type (figure 6.9). It is possible that mutation of the promoter regions may cause disruption of the binding of these NAC transcription factors, however neither of these transcription factors are members of subgroup III and so it is impossible to draw conclusions about NAC subgroup III binding.

6.2.3.4. Validation of the putative NAC-III motif using a protoplast transactivation assay

In all experiments, there appeared to be no change in binding of NAC subgroup III proteins to the promoter region. However, the high association of NAC subgroup III protein binding and the presence of the NAC-III motif still suggests that the motif is important for function. NAC proteins have been observed to promote transcription in concert with other transcription factors (Tran *et al.*, 2007), therefore it was suggested that a secondary protein might bind to the novel motif and contribute to NAC activity *in vivo*. The NAC transcription factors ANAC018, ANAC025, ANAC056 and ANAC102 have been shown to drive expression of a *GUS* reporter from Y1H-37 in a protoplast transactivation system earlier in this thesis. This was used to test the mutated Y1H-37A.

No difference was observed between the wild type and mutated forms of Y1H-37 (figure 6.10), suggesting that the NAC-III motif is not necessary for activation of transcription by subgroup III NAC proteins. It is still possible a secondary protein binds to this motif and facilitates NAC action, however this does not appear to affect the induction of transcription by NAC proteins.

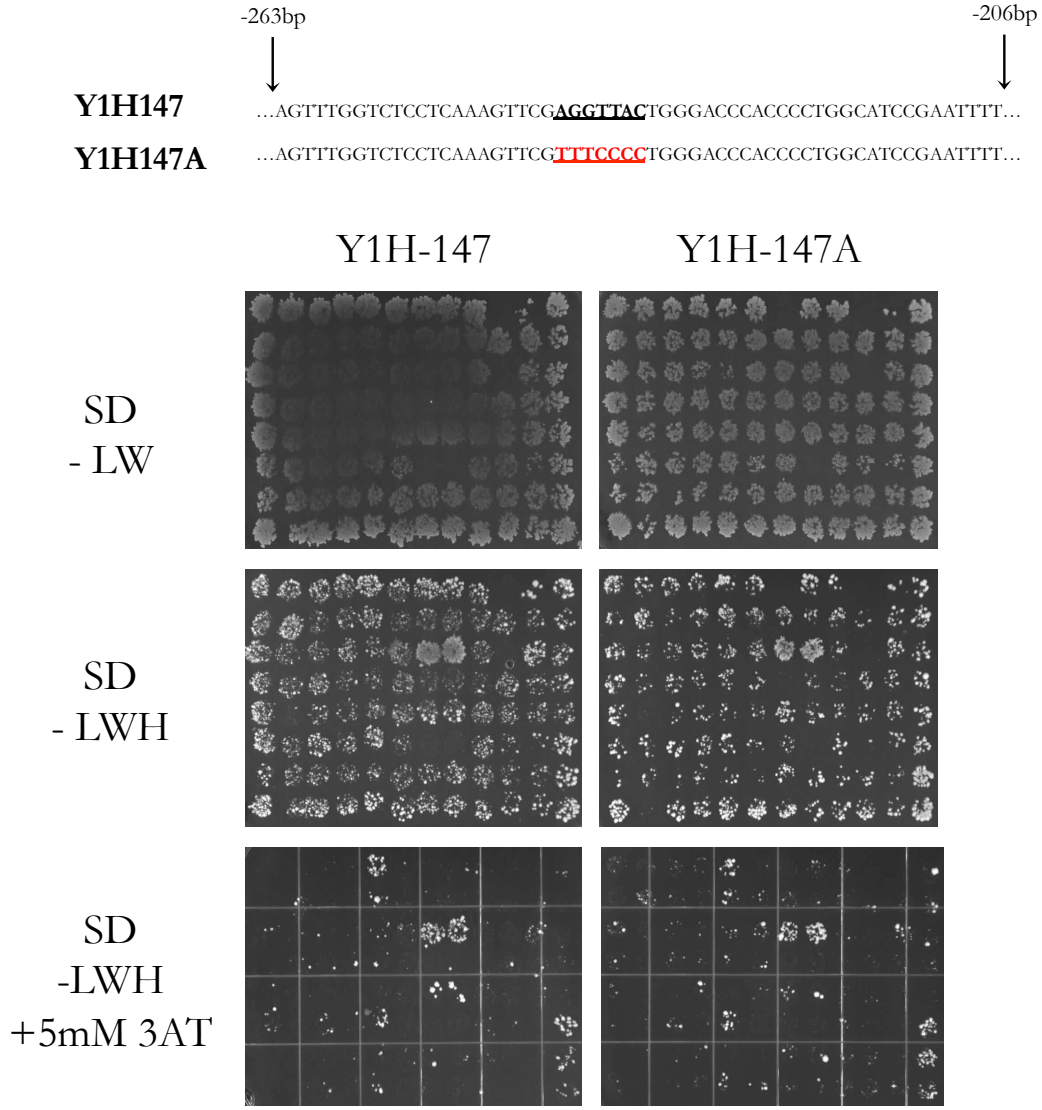


Figure 6.7: **Yeast 1-hybrid of wild type and mutated Y1H-235** Y1H-147A has a mutation in the putative NAC-III motif. The wild type and mutated promoter regions were compared for NAC binding in yeast 1-hybrid. SD minus leucine and tryptophan is selective for correct mating, while SD minus leucine, tryptophan and histidine is selective for *HIS3* expression. 5mM 3-aminotriazole was used as to suppress autoactivation of the promoter region. There do not appear to be differences in the binding characteristics of the two promoter regions.

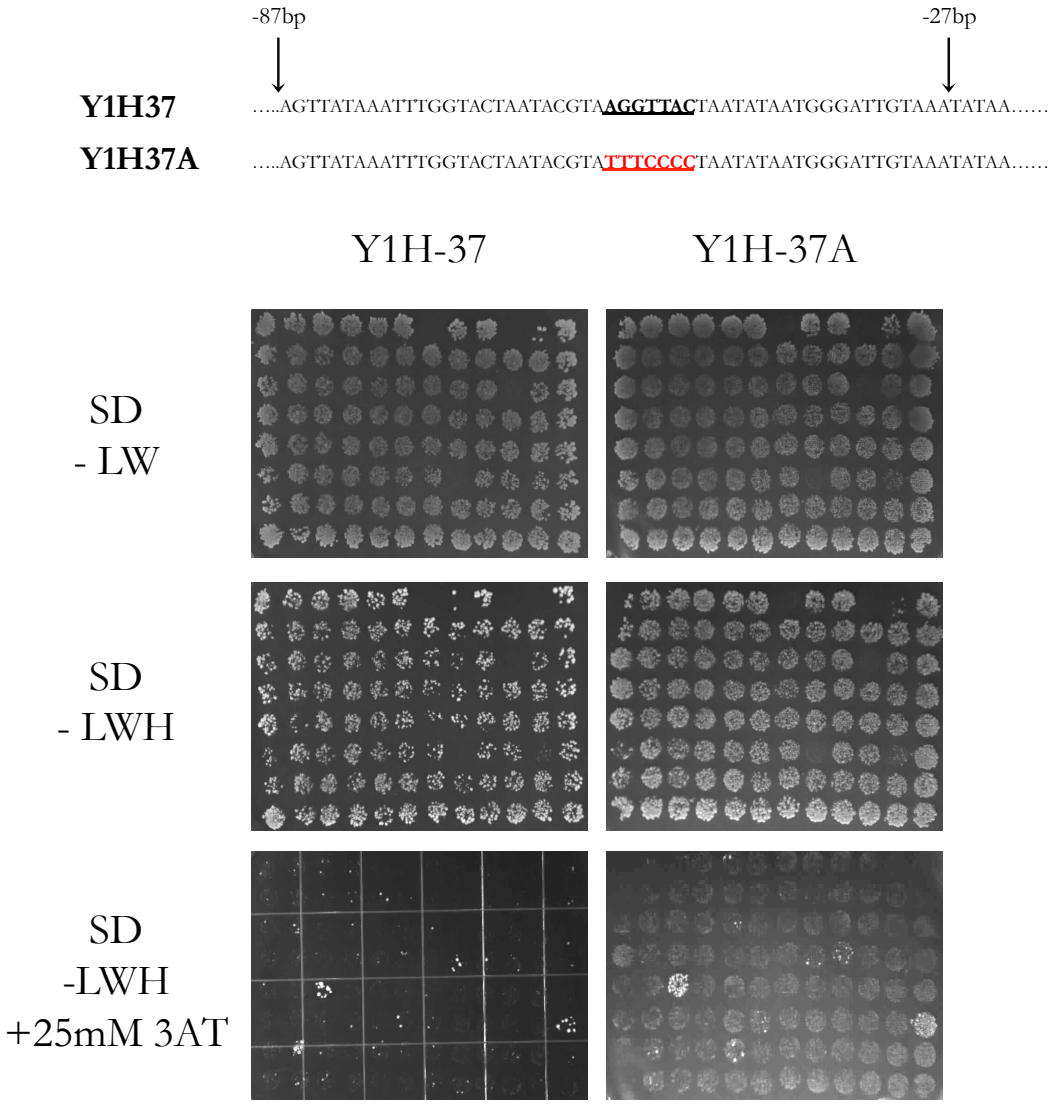


Figure 6.8: **Yeast 1-hybrid of wild type and mutated Y1H-37** Y1H-37A has a mutation in the putative NAC-III recognition site. The wild type and mutated promoter regions were compared for NAC binding in yeast 1-hybrid. SD minus leucine and tryptophan is selective for correct mating, while SD minus leucine, tryptophan and histidine is selective for *HIS3* expression. 25mM 3-aminotriazole was used as to suppress autoactivation of the promoter region. There do not appear to be differences in the binding characteristics of the two promoter regions.

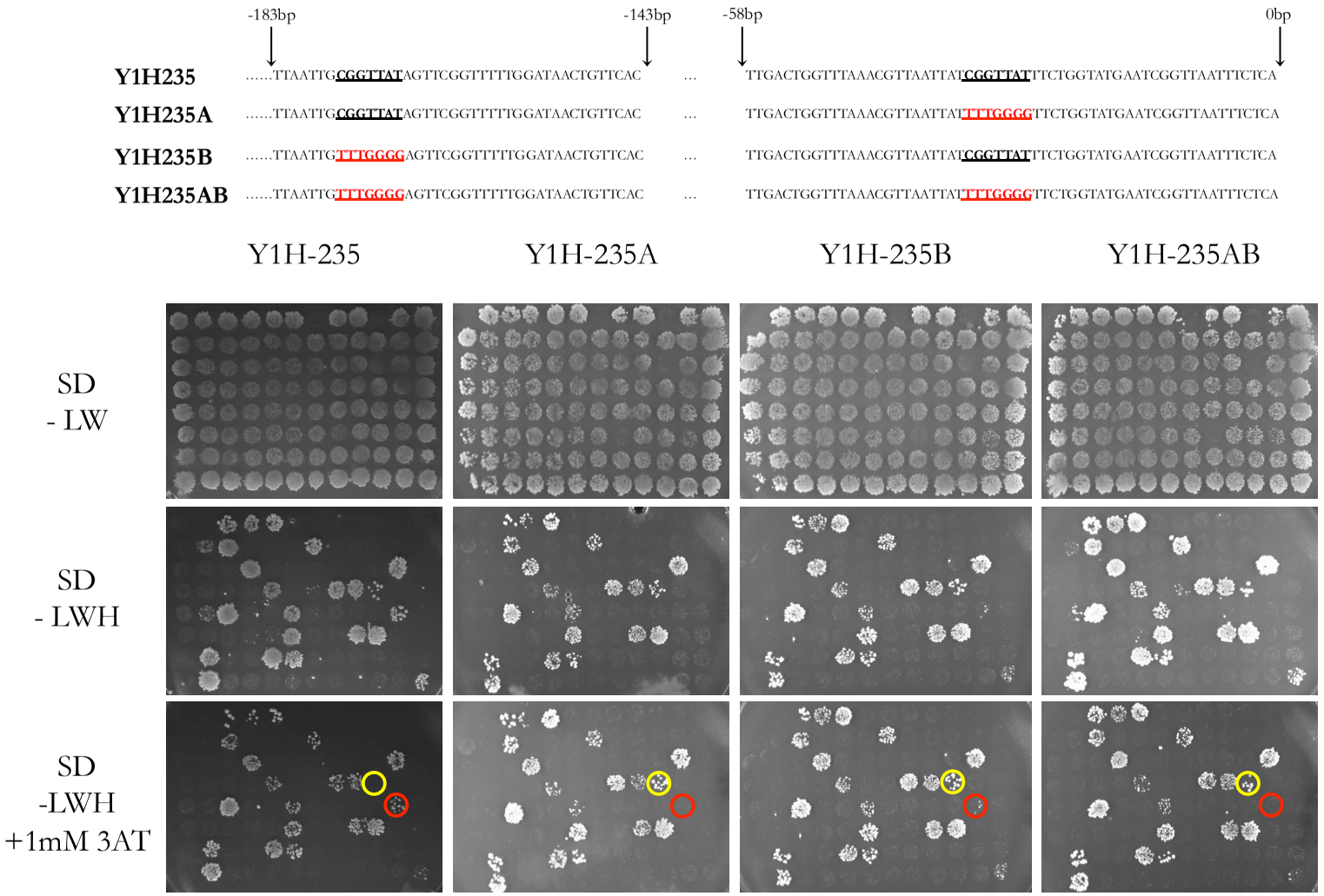


Figure 6.9: **Yeast 1-hybrid of wild type and mutated Y1H-235** Y1H-235A and Y1H-235B have had one instance of the NAC-III motif mutated, while Y1H-235AB has had both instances mutated. The wild type and three altered promoters were analysed for NAC transcription factor binding in yeast 1-hybrid. SD minus leucine and tryptophan is selective for correct mating, while SD minus leucine, tryptophan and histidine is selective for *HIS3* expression. 1mM 3-aminotriazole was used as to suppress autoactivation of the promoter region. Limited differences were observed between all three samples, however ANAC1 1 appeared to bind to the wild type but not the mutant (red circle), while ANAC 9 appeared to bind to the mutant Y1H-235 promoters, but not the wild type promoter (yellow circle).

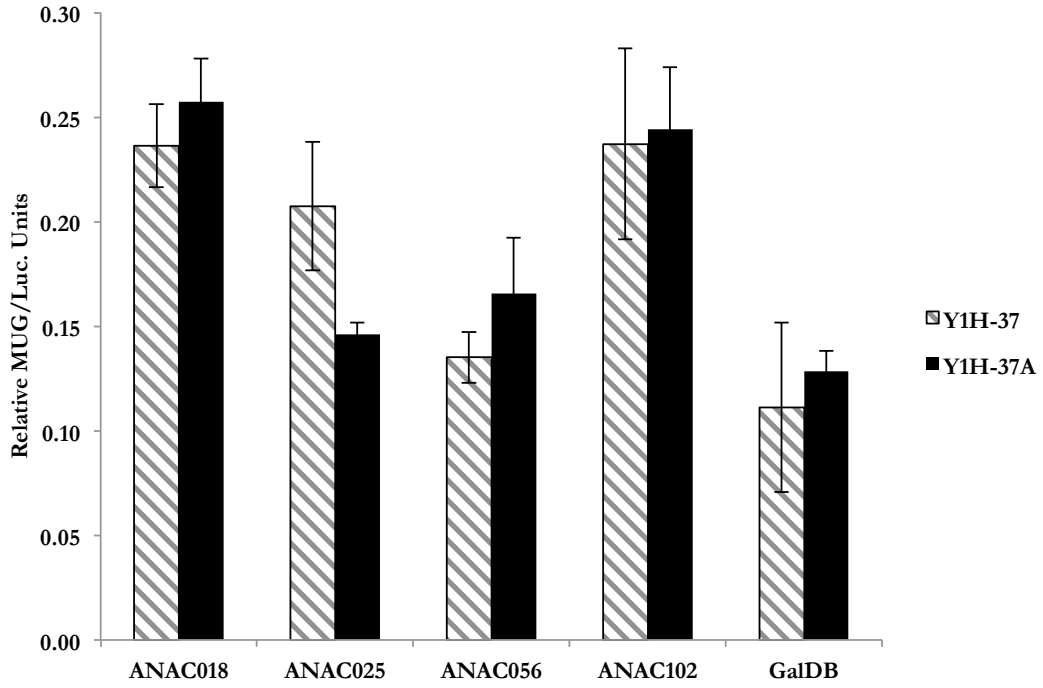


Figure 6.10: **Protoplast transactivation assay of wild type and mutated Y1H-37** ANAC018, ANAC025, ANAC056 and ANAC102 were constitutively expressed in protoplasts alongside a *GUS* reporter driven by wild type and mutated Y1H-37, a promoter region of *ANAC092.* The mutated form of Y1H-37 has a disrupted putative NAC-III motif. GalDB is used as a negative control of activation. There appears to be no difference in expression of *GUS* between the wild type and mutated Y1H-37 driven by the transcription factors.

6.2.3.5. Potential role of putative NAC-III recognition site

The role of the NAC-III motif remains unclear. It is highly conserved in the promoters that recruit subgroup III NAC proteins, yet does not appear to alter the binding of these proteins (figure 6.7, 6.8 and 6.9). Similarly, these proteins do not appear to require this motif for NAC induced transcription in a protoplast transactivation system (figure 6.10). It was decided to identify every instance of this motif in the Arabidopsis genome and investigate any shared functions of these genes.

The 500bp upstream region of every coding sequence in the Arabidopsis genome (TAIR8) was scanned for the presence of a AGGTTAC sequence using the *in silico* expression analysis tool in PathoPlant (Bolívar *et al.*, 2014). This tool retrieves all promoter regions that contain the DNA sequence defined in the input, then analyses publicly available microarray data for overrepresented patterns of expression. Although focused on plant-pathogen interactions, PathoPlant includes expression data for a number of other stresses such as cold and drought treatment that are available as part of the AtGenExpress data (Kilian *et al.*, 2007).

Stimulus	Mean induction factor	p-value	BH (FDR) adjusted p-value
X. campestris	1. 88	6.57E- 5	9.52E-03
Drought-stressed shoots 1 hr	1. 79	7.52E- 4	3.64E-02
P. syringae pv. phaseolicola 2 hpi	1. 61	6.27E- 4	3.64E-02
E. orontii 2dpi	-1. 58	1.15E- 3	4.17E-02
Cold-stressed shoots 1 hr	1. 74	2.57E- 3	5.33E-02
Cold-stressed shoots 3 hr	1. 72	2.34E- 3	5.33E-02
TMV infected leaves 4 dpi	1. 44	2.27E- 3	5.33E-02

Table 6.7: **Coexpression of genes harbouring the NAC-III motif in their promoter regions** 1 14 genes which contained the NAC-III motif in their 500 bp promoter regions were compared for coexpression across a range of publicly available microarray datasets using the *in silico* expression analysis tool in PathoPlant (Bolívar *et al.*, 2014).

1014 Arabidopsis genes contained the 5'-AGGTTAC-3' motif in their 5 bp promoter region. By comparison, the 5'-GCCGCC-3' box which is recognised by ORA59 (Zarei *et al.*, 2011) is present on the 500bp promoter regions of 1457 genes, while the AtHB recognition motif 5'-CAATNATTG-3' appears in 569 promoter regions. As such, the 1014 promoters containing the NAC-III motif seems in line with occurrence of a functional transcription factor motif. In addition, the *in silico* analysis by PathoPlant determined that there was an overrepresentation of genes that are increased in expression during *X. campestris*, *P. syringae* and *E. orontii* infection, as well as drought stress (table 6.7). This may suggest the motif is associated with a range of biotic and abiotic stress responses. No GO terms were overrepresented with the 1014 genes that contained the NAC-III motif in their promoter (as determined by BiNGO, Maere *et al.*, 2005).

This may indicate the motif is not associated with one particular condition or function. Instead, the motif is present on many promoter regions and helps regulate their transcription level in association during multiple stresses. How this motif regulates gene expression remains elusive.

6.3. Discussion

6.3.1. Use of high-throughput yeast 1-hybrid for detection of new transcription factor recognition sites

The NAC transcription factors are one of the largest families of transcription factors in Arabidopsis (Ooka *et al.*, 2003). They are present in all identified plants and have even been suggested to be responsible for the adaptation of plants to a land-dwelling lifestyle (Xu *et al.*, 2014). They regulate an enormous range of functions, covering nearly every aspect of development and stress response (Olsen *et al.*, 2005b; Lindemose *et al.*, 2013; Jensen & Skriver, 2014). Identification of their recognition sequence is one of the key aspects to understanding their direct targets, however only a small number have been studied in depth.

Since the NAC family is a large family of transcription factors and regulates such a large number of processes, it seems unlikely that there is one NAC recognition sequence for all NAC proteins, therefore attempts have been made to use high-throughput techniques to analyse NAC binding on a large scale (Olsen *et al.*, 2005b; Lindemose *et al.*, 2014). However, these studies have been limited to a relatively small number of NAC transcription factors due to experimental technique, therefore they can never identify a NAC recognition sequence for all NAC proteins simultaneously. Here, the analysis of all NAC transcription factors simultaneously has been attempted using yeast 1-hybrid.

Yeast 1-hybrid lends itself to this study. Modern cloning systems such as Gateway allow for easy and quick manipulation of many cloned genes and promoter regions, which is very adaptable to yeast 1-hybrid (Deplancke *et al.*, 2004). Furthermore, unlike many other DNA:protein analysis techniques, no expensive purification or reagents are required for each result, meaning yeast 1-hybrid is considerably cheaper than many other techniques. As such, yeast 1-hybrid can be done on a scale limited only by time and labour constraints. Yeast 1-hybrid facilitated by robotic manipulation and quantification equipment could negate these constraints (Pruneda-Paz *et al.*, 2014), facilitating the analysis of even more promoter regions.

Compared to techniques such as protein binding microarrays, yeast 1-hybrid does have a number of limitations. Although PBMs are limited to a single transcription factor at a time, they can analyse the simultaneous binding of that transcription factor to many thousands of DNA sequences simultaneously in an unbiased manner (Berger & Bulyk, 2009). As such, DNA sequences produced are relatively statistically robust, as they have many thousands of results to support the motif. Conversely, yeast 1-hybrid is inherently limited in the number of DNA sequences that can be analysed because each promoter region analysed requires an individual experimental replicate. Therefore, yeast 1-hybrid experiments are limited to small numbers of DNA sequences, which in turn reduces any the statistical validity of new motifs produced.

6.3.2. Comparison to known NAC recognition sites

In this study, a number of transcription factor motifs associated with particular clades of NAC transcription factors were identified. For example, subgroup I and II appeared to both recognise a C/T rich motif. Subgroup I includes the NTL group of membrane bound NAC transcription factors, while subgroup II contains *ANAC092* and *VND3/ANAC105* amongst others. Interestingly, these two subgroups were shown to recognise different binding sequences using PBMs in Lindemose *et al.* (2014), however in this study the motifs are relatively similar. These differing results may be due to the difference between the two techniques. Yeast 1-hybrid represents real binding in an active eukaryotic cell, compared to the *in vitro* PBM system. This may mean the results from yeast 1-hybrid are more applicable to how the transcription factor(s) interact with DNA *in planta*. However, PBMs produce a much larger amount of data which can produce more statistically robust results. Therefore it is difficult to say which is more accurate, although it is entirely possible that both are equally valid. It may be that NAC proteins bind to certain DNA sequences in an *in vitro* system, but when placed in the context of living tissue they bind to slightly different sequences, due to the influence of the intracellular conditions. For example, *in vivo*, the primary role of a transcription factor is to promote the association of RNA polymerase with DNA in order to facilitate transcription. This requirement is removed during an *in vitro* technique such as PBMs, therefore there may be a number of positive results that do not necessarily correlate with true promotion of transcription.

The 'core' NAC recognition sequence CGT[G/A] has been reported for many NAC transcription factors (Jensen & Skriver, 2014). In this study, the CGT[G/A] sequence was not detected as enriched in promoter regions that recruited NAC proteins. This may highlight that NAC proteins do not just recognise a CGT[G/A] motif, instead recognising longer DNA sequences that incorporate the CGT[G/A] as part of their sequence. Indeed, since CGT[G/A] is very short motif and appears regularly in genomic DNA, it is likely that the auxiliary sequences or flanking regions moderate NAC proteins binding to the CGT[G/A] sequence, therefore conferring different binding specificities to NAC proteins. Perhaps, CGT[G/A] is not as effective at recruiting NAC proteins in yeast 1-hybrid as it is in other techniques, therefore CGT[G/A] was not detected as a highly conserved motif. Alternatively, the short size of a CGT[G/A] sequence meant that MEME never regarded it as statistically significant, since length of sequence is a factor in determining the validity of a motif.

Interestingly, a double-thymine sequence was frequently present in the DNA sequences detected. TT was present in the motif determined for subgroup I, II and III. TT has been a core sequence of many NAC binding sites identified so far. For example, ANAC092 recognises the sequence TTnCGT (Matallana-Ramirez *et al.*, 2013), while TT is adjacent to the binding site of ANAC019 (Jensen *et al.*, 2010; Welner

et al., 2012). In the crystal structure of an ANAC019 dimer binding to DNA, the TT sequence sits between the two NAC residues (Welner *et al.*, 2012). Perhaps, the TT sequence facilitates formation or binding of NAC dimers to DNA. With shorter sequences such as those seen on PBMs, this may be missed because the sequence is not long enough to incorporate a full binding site either side of a TT residue. In yeast 1-hybrid, the presence of a TT sequence in a larger section of DNA could facilitate the formation of NAC transcription factor dimers. As such, it is possible TT is a key component in many NAC binding sites, but as a small, simple sequence is often overlooked. Perhaps it is one of the most crucial sequences for NAC binding.

6.3.3. The putative NAC-III sequence

A novel motif was highly conserved in the promoter regions that subgroup III NAC proteins recognised. This motif, 5'-AGGTTAC-3', was present in all the promoters that NAC subgroup III proteins recognised, but not in the promoter regions that were bound by other NAC proteins. Therefore it was suggested that this motif was responsible for recruiting the NAC subgroup III proteins. However, mutation of this motif did not appear to alter binding or NAC driven transcriptional activity in protoplasts. Therefore it is not clear why this motif is tightly associated with NAC subgroup III binding. Perhaps, it recruits a non-NAC protein to the promoter region, which facilitates NAC subgroup III binding, therefore allowing NAC proteins to bind and/or drive transcription. However it is unlike any previously known transcription factor motif, plus its presence on the promoter was not associated with a particular molecular function in *Arabidopsis.* Therefore, the role of the NAC-III motif remains uncharacterised and needs further work.

6.3.4. Yeast 1-hybrid could offer an alternative method for determination of transcription factor recognition sequences

There are many techniques for *de novo* DNA recognition site determination, such as SELEX, PBMs and ChIP-Seq, however these are all limited by the number of transcription factors that can be studied. Here, yeast 1-hybrid has been used to simultaneously analyse the binding of 94 NAC family transcription factors to DNA. Although not successful in confirming a new NAC transcription factor motif, this technique has been able to detect new motifs associated with NAC binding. However, the limited number of promoter regions (30) combined with the long (400-500bp) DNA sequences are two areas which could easily be improved. If a larger number of promoter regions were screened, each new motif might have more DNA sequences matched to it, therefore the consistency of individual residues in a transcription factor motif could be clarified, which should correlate with their importance (or lack thereof). Similarly, aligning long DNA sequences often causes irrelevant DNA sequences to be aligned, leading to false positive results. As such, reduction in target

promoter regions could reduce the rate of false positives and improve validity. A good novel design could involve the production of synthetic promoter regions, that show limited homology except in the essential areas. Comparison of these promoter regions could lead to identification of many new recognition sequences for NAC proteins.

Nevertheless, this study represents a new technique for defining NAC transcription factor recognition sequences using yeast 1-hybrid. Unlike other techniques, yeast 1-hybrid can be used to analyse the binding of a large number of transcription factors simultaneously, which makes it especially pertinent to plant species which have highly expanded gene families. As such, development of high-throughput techniques such as this are required to help resolve DNA-binding characteristics of transcription factors in plants.

7. General Discussion

7.1. The *ANAC092* regulatory network represents an example of the highly interconnected signalling network that regulate Arabidopsis stress responses

As sessile organisms, plants are subject to an enormous range of environmental stresses that they cannot move away from. However, they are not passive and in response to these stress conditions they can induce a variety of adaptations. Gene expression changes are at the core of this stress response, facilitating the production and synthesis of new cellular machinery to adapt to the new conditions. The mechanisms by which these large scale transcription events are regulated is one of the key targets of plant and systems biology.

Many stress responses are interwined in Arabidopsis. Some stress response mechanisms are broad acting and generic, restricting growth and development in favour of resilience and survival (Huot *et al.*, 2014), while others are highly specific, activating a particular response only applicable to that stress (Thomma *et al.*, 2011). Ensuring the appropriate biochemical and physiological change is induced in response to each stress condition is one of the key attributes of plant stress signalling. Underlying this stress signalling is the vast and highly cross-linked transcriptional network, composed of the dramatically expanded Arabidopsis transcription factor families, which form an extensive network of signals that transduce stress recognition to appropriate adaptation.

Like many biological networks, gene regulatory networks in Arabidopsis are examples of scale-free networks (Albert, 2005). That is to say, the expression of a small number of genes directly corresponds to the expression of a disproportionately large number of other genes. The most highly connected genes are often known as 'hubs', or 'master regulators', due to their enormous influence on downstream transcriptional changes (Kaufmann *et al.*, 2010). It is these genes which cross-link a number of stress responses, thereby connecting the stress response network. One of these genes in Arabidopsis is the NAC transcription factor *ANAC092.*

7.1.1. ANAC092 is a functional transcription factor during *Botrytis cinerea* infection

In this thesis, a known promoter of leaf senescence (Oh *et al.*, 1997) and stress response (Balazadeh *et al.*, 2010a; Kim *et al.*, 2011) has been shown to act as a functional gene during *Botrytis cinerea* infection. The *ANAC092* transcript level dramatically increased during Botrytis infection, while Arabidopsis lines deficient or enhanced in *ANAC092* expression have altered phenotypic responses. Visible symptoms and gene expression changes indicate ANAC092 may promote senescence-like processes in the vicinity to the lesion site, suggesting that ANAC092 conserves its role in promoting senescence in the area around the infection.

However, the positive correlation of *ANAC092* expression levels and Botrytis lesion size leaves a questions unanswered. If ANAC092 positively contributes to the spread of a *Botrytis cinerea* lesion, then why does is ANAC092 expression triggered during a Botrytis infection? It would be advantageous for the plant to reduce the spread of the lesion, therefore expression of *ANAC092* is detrimental to resisting the disease.

Hypothetically, it is possible that processes such as cellular degradation and nutrient recycling are being used to reclaim nutrients in the vicinity of the lesion, thus *ANAC092* expression is induced as part of a strategy to withdraw nutrients and macromolecules away from the lesion site. A number of senescence related genes are expressed during Botrytis infection (Windram *et al.*, 2012), while pathogen response genes are also expressed during senescence (Quirino *et al.*, 1999). It has even been suggested that senescence has evolved as a strategy to combat pathogen infection (Wu *et al.*, 2012b). Therefore, perhaps ANAC092 is a positive regulator of biotic stress responses, but a deterministic measure of phenotype such as lesion size does not capture the relationship between pathogen and host. Perhaps, experiments on Botrytis susceptibility of the whole plant, as opposed to detached leaves could help elucidate whether ANAC092 contributes to continued survival of the whole organism.

Alternatively, host senescence is induced as an infection mechanism by *Botrytis cinerea*. Botrytis has previously been observed to induce senescence (Swartzberg *et al.*, 2008), presumably to induce senescence associated cell-death and thus spread. Perhaps this exploitation of host mechanisms may function through ANAC092. However, if this is the case, why has Arabidopsis not adapted to prevent *ANAC092* induction during Botrytis infection? Experiments using related, but distinct necrotrophic species such as *Trichoderma harzianum* may help determine whether infection dependent ANAC092 expression is a Botrytis-specific phenomenon.

7.1.2. Analysis of the *ANAC092* promoter using high-throughput yeast 1-hybrid

In an attempt to understand the regulatory mechanisms underpinning *ANAC092* expression levels during multiple stress conditions, a high-throughput yeast 1-hybrid

technique was employed. A number of transcription factors were observed to interact with specific regions of the *ANAC092* promoter, thus indicating they may be responsible for regulating *ANAC092* transcription. Each region of the *ANAC092* promoter appeared to recruit particular transcription factor families, indicating that specific proteins recognise short sequences in the larger *ANAC092* promoter. Furthermore, these proteins were not only from the same family, but often the same clade, indicating the proteins have the binding capability to resolve small differences in DNA. However, it is important to note that this is not a comprehensive analysis of the *ANAC092* promoter region, as techniques such as yeast-1 hybrid are expected to have a high-rate of false negatives. While it is difficult to establish an actual baseline rate, lack of plant cellular environment and auxiliary proteins are expected to impede many transcription factor:DNA interactions. In yeast, comparisons between ChIP-seq, protein binding microarrays and *in silico* motif scanning has revealed that while both experimental techniques corroborate with *in silico* motif scanning, there is limited overlap between wet-techniques (Mirzaei *et al.*, 2013). Yeast 1-hybrid was not included in this comparison, due to the inherent difficulties in studying transcription factors in the host system, however, it is likely that experimental techniques show distinct results, rather then overlapping ones.

Use of a protoplast transactivation system allowed for rapid testing and validation of yeast 1-hybrid results, showing that particular transcription factors induced expression of *ANAC092* through a reporter construct without the requirement to transform Arabidopsis. As such, the majority of the transcription factors seen to bind to the *ANAC092* promoter could be tested with relative ease. The use of a protoplast system had two net results. Firstly, it supported the yeast 1-hybrid results in a plant intracellular environment. Secondly, it showed transcription roles for each of the confirmed regulators of ANAC092. The results obtained indicated that expression was promoted by members of the NAC and MYB transcription factor families, but repressed by AtHB proteins, suggesting different transcription factor families may have differing roles with regards to *ANAC092* regulation.

During these experiments, a high-throughput variant of the protoplast system was adapted. Here, the development of a high-throughput platform for protoplast transactivation studies was used to generate a high-number of replicates, however in the future applications could be extended to analysis of many transcription factors simultaneously. Alternatively, this could be extended to other areas of study such as protein heterodimerisation using FRET-FLIM (Bücherl *et al.*, 2014) or BiFC (Walter *et al.*, 2004).

One aspect that has not been addressed in this thesis is the action of transcription factor combinations. Previously, it has been shown that certain transcription factors are ineffective when binding to DNA in isolation, but have a potent transcriptional activation capacity when binding as a heterodimer with a secondary protein (Tran *et al.*, 2007). It is possible that the transcription factors that bind to the *ANAC092*

promoter act synergistically or antagonistically with each other, thus generating another layer of regulation for *ANAC092* transcription at the promoter. Further work could analyse the protein:protein interactions between the putative regulators of *ANAC092,* or the inhibitory action of transcription factors occupying DNA binding sites and preventing other transcription factors accessing the promoter. Finally it is possible that proteins that are unable to bind to the *ANAC092* promoter also have a role in the transcription factor:DNA interactions observed here. For example, inhibitory proteins could bind to a transcription factor and prevent it binding to the *ANAC092* promoter, before being released upon a signal. This would add a new mechanism by which transcription of *ANAC092* could be triggered. Interactions such as this could be studied through yeast 2-hybrid or proteome analysis techniques.

7.1.3. Integration of multiple datasets is critical for analysis of gene regulatory networks

In this project, a combination of molecular biology, timeseries expression data and network inference was used to construct a gene regulatory network for *ANAC092* (figure 7.1). The network is not fully validated, but it may highlight key features of *ANAC092* regulation that can be expanded further. In particular, the separate topologies of the gene regulatory network for different stress conditions is an interesting concept that could be explored further, perhaps by integration with other stress conditions or phytohormone signalling such as ethylene and jasmonate response pathways.

The incorporation of multiple datasets is a key element to building robust and fully validated gene regulatory networks. The use of complementary techniques can support or modify existing hypotheses. In this study, the lack of context in a yeast 1-hybrid experiment and protoplast transactivation system are compensated by the gene expression profiles created as part of the PRESTA project. This theory was used to predict a gene regulatory network for *ANAC092* active during *Botrytis cinerea* infection or age-induced senescence.

Analysis of the gene expression data suggested that particular transcription factors appeared to regulate *ANAC092* during Botrytis infection, age-induced senescence, or both. Initally, analysis of timeseries data demonstrated that certain transcription factors exhibited similar expression profiles as *ANAC092* during one or more stress conditions. This suggested that the expression of these genes were linked, possibly through regulation. hCSI was used to provide a robust statistical methodology behind this form of hypothesis generation, however, it seemed to show limited success. No putative regulators of *ANAC092* were suggested to be responsible for *ANAC092* transcription, despite clear differences in the expression profiles. This possibly implies that hCSI was not an appropriate network inference technique for this particular network. Exactly why this is the case is not clear, especially consider-

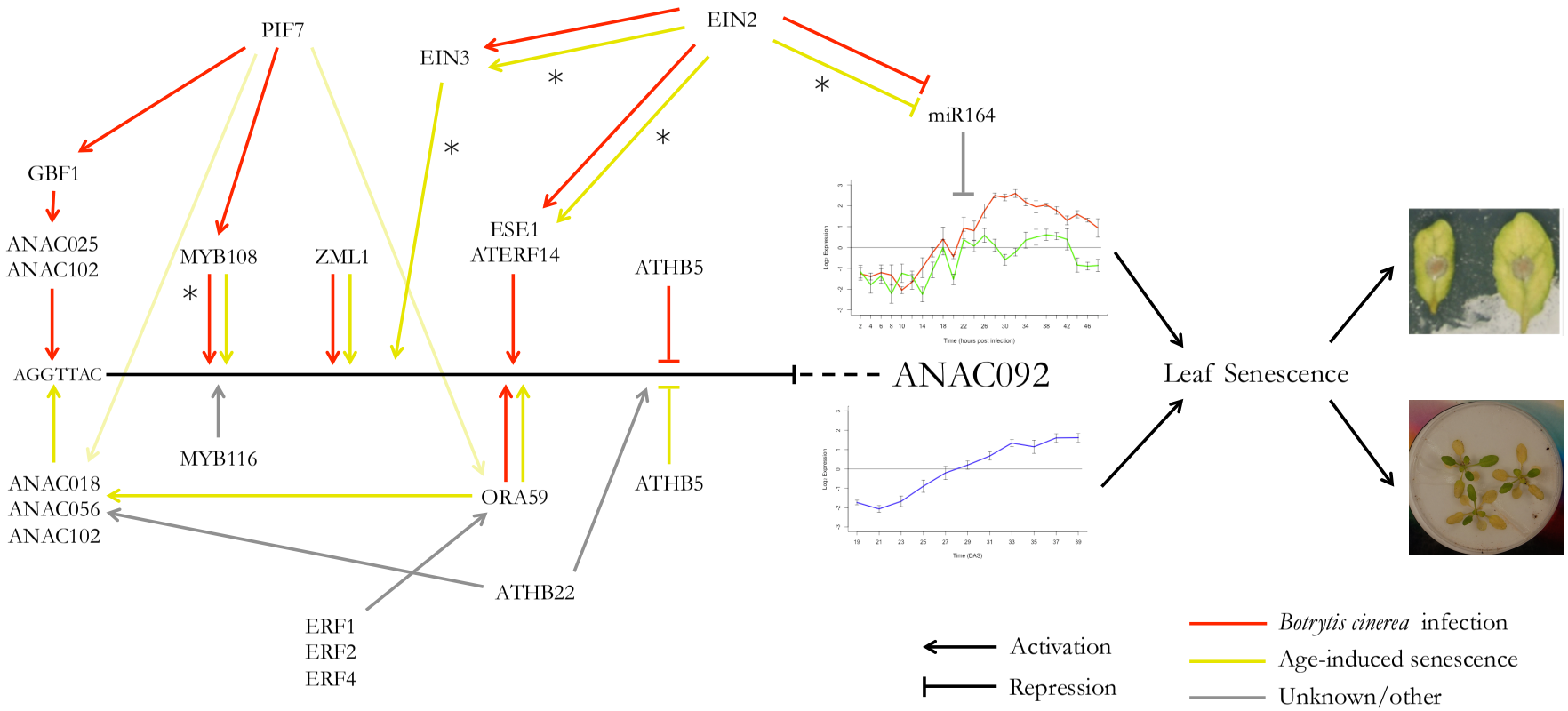


Figure 7.1: **Summary of thesis findings** Diagram of results from this thesis, showing methods of transcriptional regulation for *ANAC092* that may occur. A gene regulatory network constructed from yeast 1-hybrid interactions, network inference and known regulatory pathways, showing mechanisms by which *ANAC092* transcription may be promoted during *Botrytis cinerea* infection and age-induced senescence. In turn, ANAC092 triggers expression of senescence associated genes which cause the onset of leaf senescence. Pictures of 35S:*ANAC092* plants, during *Botrytis cinerea* infection (top) and dark-induced senescence (bottom). * indicates validation of an interaction in literature or this work.

ing this network inference technique has previously been used for other transcription factor based networks from Arabidopsis (Penfold *et al.*, 2012). It may be the case that in certain situations, use of network inference techniques are inappropriate and may not be informative. As such, validation with an alternative methodology such as experimental data is critical.

The implication from the timeseries data was that while the promoter region of *ANAC092* can recruit a number of proteins, only a subset of these bind to the promoter during each stress. In this manner, there may be two distinct protein multimers that form around the *ANAC092* promoter region during the two stresses. There are many examples of transcription factors only binding to DNA during a particular context, but very few examples of distinct transcription factors binding to the same promoter under different contexts.

In this project, validation of these interactions was conducted using transgenic plants, primarily knock-outs and overexpressors. Experiments using plants constitutively expressing transcription factors are confounded by the strong phenotypic affects that can be induced. In comparison, knock-out plants are expected to only be affected when the gene is expressed at a high level, such as during the rapid induction of stress responsive genes. On the other hand, effects in knock-out plants can be small due to functional homology and compensating stress signals in the plant. In yeast, perturbation of genes encoding transcription factors only affects 3-5% of the downstream targets detected in ChIP-chip experiments (Haynes *et al.*, 2013), which suggests that functional knock-outs are poor measures of true regulation. Use of synthetic biology could offer an excellent alternative to these techniques, for example, an auto-regulating gene that induces a positive feedback loop of its own expression could cause rapid constitutive expression during a stress which can be detected in downstream gene expression changes.

A common methodology when studying regulation is to manipulate the regulator gene expression and detect changes in target gene expression, however, at a certain level results such as this are inferred. When combined with *in vitro* evidence for DNA binding, the correlation of gene expression changes in parent and child is presumed to be indicative of a functional affect. However, this is not direct evidence, i.e. there is no indication of *in vivo* DNA binding during stress, as there would be in a ChIP based experiment. This is largely because it is very difficult and time consuming to see direct evidence for DNA binding *in vivo*, especially when studying low abundance transcription factors. New techniques are being developed continuously and it is possible live *in vivo* DNA binding may become a common technique in the future. In yeast, developments are being made to study the dynamic proteome that forms around the transcription initiation site using highly sensitive mass spectrometry (Mirzaei *et al.*, 2013), while in humans live *in vivo* imaging of a single polymerase complex during transcription has been observed (Revyakin *et al.*, 2012) In plants, cell cultures expressing libraries of tagged transcription factors are facilitating the

development of high-throughput *in vivo* DNA binding experiments (Verkest *et al.*, 2014), which may help create a genome wide gene regulatory network.

7.1.4. The DNA recognition site of NAC transcription factors

Transcription factor binding site profiles are beginning to approach complete coverage in humans (Jolma *et al.*, 2013), but the massively expanded transcription factor families in plants (Shiu *et al.*, 2005) means coverage in Arabidopsis is comparatively poor. For example, in Arabidopsis the MYB transcription factor family has 190 members (Dubos *et al.*, 2010), while there are six in fly, three in worm and ten in yeast. This has meant comprehensive analysis of transcription factor binding is limited to a subset of each protein superfamily, which are used as representatives for the others. While it is true structural conservation in transcription factor proteins can confer similarities in DNA binding (Yamasaki *et al.*, 2013), individual specificity cannot exist when all transcription factors recognise the same region. Therefore it is likely that each clade transcription factor has its own binding sequence, that is related, but not identical to the binding sequence of its close relatives.

In this thesis, high-throughput yeast 1-hybrid was used to characterise the DNA binding of NAC transcription factors. Specific subclades were seen to recognise particular DNA sequences, implying that related proteins may recognise similar DNA sequences and therefore recognition is a function of the amino acid sequence. However, it was not possible to validate these motifs. Nevertheless, when confronted with the enormous number of transcription factors in plants, analysis of DNA binding domains may need to move towards a high-throughput methodology, scanning a high proportion of each family for DNA binding, instead of using particular transcription factors as models for the entire gene family.

Alternatively, applying reductionist thinking to transcription factor:DNA interactions may be too limited. One simple motif may not be responsible for recognition, instead the interaction of a protein on DNA may be guided by a number of localised conditions that induce or repress formation of a larger complex. There are comparisons to protein folding, which was initially seen as a simple factor of amino acid sequence conferring a secondary and tertiary structure, but was then shown to be dependent on other proteins, post-translational modifications and many more aspects. Transcription factors may be dependent on conditions such as cofactors, DNA secondary structure and the influence of other transcription factors. Perhaps DNA binding of a NAC transcription factor is dependent on the influence of other protein:DNA interactions, more then the recognition of a small DNA sequence.

7.1.5. Gene regulatory networks

The analysis of gene regulatory networks is one of the most fundamental aspects of biological research, encompassing every adaptive process that can occur in an

organism. Studying gene regulatory networks requires high-throughput molecular biology, bioinformatics and mathematical modelling, in addition to many other techniques. In this thesis, genome wide expression data and molecular biology has led to construction and analysis of a regulatory network for expression of *ANAC092*. This network may go some way to explaining expression levels of *ANAC092* during developmental senescence and *Botrytis cinerea* infection. While small and limited, the network here may highlight some of the key aspects of regulation of *ANAC092* during multiple stress conditions. Like all models, this network is wrong, as it lacks many of the stresses conditions and tissue types where ANAC092 is expressed, however, it is useful and can indicate key aspects such as major transcription factors and differential binding. The methodology can be applied to other regulatory networks, and as such, in conjunction with developments in molecular biology and modelling techniques, it is possible more multi-stress networks will be resolved in plants and other species.

A. Methods and Materials

A.1. Primer sequences

Function	Sequence (5'-3')
ANAC092 qPCR forward primer	TGCCGATGGTACAAAGGTTC
ANAC092 qPCR reverse primer	TTTCTTGGTCGGAGAAGCAG
PUX1 qPCR forward primer	TTAGAGGCCATGACCAAAGC
PUX1 qPCR reverse primer	TCTGTGGATTTACGCTCGTG
ANAC018 qPCR forward primer	GAGCAGTTGTTTCCACCGGTGA
ANAC018 qPCR reverse primer	ACGGAGGTGGAAGTTACTCGATG
ANAC025 qPCR forward primer	TTTCGGTTTCACCCGACTGAT
ANAC025 qPCR reverse primer	TCCACTTCCAGTCTCAATCATCGC
ANAC056 qPCR forward primer	GGAACTTCCCGCTAAAGCATCG
ANAC056 qPCR reverse primer	CAGAGCGGCGACTTCAGGTTAT
SAG12 qPCR forward primer	AGGCACATCGAGTGGATGAC
SAG12 qPCR reverse primer	TCAATGCGTTCGACGTTGTT

Table A.1: **Primers used in qPCR** Table of primers used in qPCR, shown as 5'-3'. All primers were from Integrated DNA Technologies (IDT).

Table A.2: **Primers designed for cloning of promoter regions** Primers used for cloning of promoter fragments for yeast 1-hybrid. Sequences are shown 5' to 3', with sequence matched to genomic sequence (upper case) and *att*B site adaptors.

Function	Sequence (5'-3')
ANAC092 promoter fragment 1 reverse primer	ggggaattcGTTGTATTAGATTTAAACGCGAAACCTCATG
ANAC092 promoter fragment 1 forward primer	ccccaagcttacgcgtTTTATCCTAATAGGGTTTCTAAAAATGATC
ANAC092 promoter fragment 2 forward primer	aaaaaagcaggctTCTCTTCAAACCATTCACACTGAATTATTATATTTAC
ANAC092 promoter fragment 2 reverse primer	caagaaagctgggtTGAGGTTTCGCGTTTAAATCTAATACAACTATC
ANAC092 promoter fragment 3 forward primer	ggggaattcATACATTGTTTTCACGAGATGGATAACATTTG
ANAC092 promoter fragment 3 reverse primer	ccccaagcttacgcgtCAATTTGACCAGGAACACTTCACCTGTAAC
ANAC092 promoter fragment 1A forward primer	aaaaaagcaggcttcCCTCAACTTTCTTCTCTCTTCTCAAAAAC
ANAC092 promoter fragment 1A reverse primer	caagaaagctgggtcTTTATCCTAATAGGGTTTCTAAAAATGATC
ANAC092 promoter fragment 1B forward primer	aaaaaagcaggcttcGAATAAATTATTTTAGTGTTCTTATTTCTC
ANAC092 promoter fragment 1B reverse primer	caagaaagctgggtcGTTTTTGAGAAGAGAGAAGAAAGTTGAGG
ANAC092 promoter fragment 1C forward primer	aaaaaagcaggcttcCTTTTTCTTTATAGTAGGTAAAACTCTTG
ANAC092 promoter fragment 1C reverse primer	caagaaagctgggtcGAGAAATAAGAACACTAAAATAATTTATTC
ANAC092 promoter fragment 1D forward primer	aaaaaagcaggcttcGTTGTATTAGATTTAAACGCGAAACCTC
ANAC092 promoter fragment 1D reverse primer	caagaaagctgggtcCAAGAGTTTTACCTACTATAAAGAAAAAG
ANAC092 promoter fragment 2A forward primer	aaaaaagcaggctGTCCATTCTTTATCTCATTATCAACG
ANAC092 promoter fragment 2A reverse primer	caagaaagctgggtTGAGGTTTCGCGTTTAAATCTAATACAACTATC
ANAC092 promoter fragment 2B forward primer	aaaaaagcaggctGTACCAAATTTATAACTCGAAAC

ANAC092 promoter fragment 2B reverse primer	caagaaagctgggtCTATTTCAAGAACTTTATTATTTC
ANAC092 promoter fragment 2C forward primer	aaaaaagcaggctTCTCTTCAAACCATTCACACTGAATTATTATATTTAC
ANAC092 promoter fragment 2C reverse primer	caagaaagctgggtGTCCATTCTTTATCTCATTATCAACG
ANAC025-1 promoter fragment 1 forward primer	aaaaaagcaggctTCTTATGCTAGTACCCAAAGTTTATACATG
ANAC025-1 promoter fragment 1 reverse primer	caagaaagctgggtCAGGTGTTAGGTTTGTGTGAGCATATGAG
ANAC025-2 promoter fragment 1 forward primer	aaaaaagcaggctTCCTATAGGATGAGGTCCACTAGATTCAATC
ANAC025-2 promoter fragment 1 reverse primer	caagaaagctgggtCCTAGTAAACTATGAAGATAATTAATAC
ANAC025-3 promoter fragment 1 forward primer	aaaaaagcaggctTCGTCTCCTCGAATCCTCTCATGGAACAG
ANAC025-3 promoter fragment 1 reverse primer	caagaaagctgggtCGCTAGGATGTTTTGTTACATTTCGGTGG
ANAC018-1 promoter fragment 1 forward primer	aaaaaagcaggctTCGATTCACGAAGATAAGATAGCATCATC
ANAC018-1 promoter fragment 1 reverse primer	caagaaagctgggtCTTTTATCCTCTGGAATCTTTTGTACGTAG
ANAC018-2 promoter fragment 1 forward primer	aaaaaagcaggctTCTAAACTCGGCTGAATTATGGGAGATTATG
ANAC018-2 promoter fragment 1 reverse primer	caagaaagctgggtCCAGGAAAAAGGAATAAAATGTCATACC
ANAC018-3 promoter fragment 1 forward primer	aaaaaagcaggctTCCTTACTATAGAGACAAAAATATATATATGC
ANAC018-3 promoter fragment 1 reverse primer	caagaaagctgggtCGTGTTTGTAATTAACAATTGTACCCC
ANAC056-1 promoter fragment 1 forward primer	aaaaaagcaggctTCATGTTTCGAAGACAAAGTGCTATTAAG
ANAC056-1 promoter fragment 1 reverse primer	caagaaagctgggtCCACAGAGTCGGCGTTACCATTCTTTTTA
ANAC056-2 promoter fragment 1 forward primer	aaaaaagcaggctTCGCTATTATGTAATTAGCAATGACTAATC
ANAC056-2 promoter fragment 1 reverse primer	caagaaagctgggtCTATAGTTACCTGCCCTAATGATTTAAG

ANAC056-3 promoter fragment 1 forward primer	aaaaaagcaggctTCTAAAATCGTATTACAGCATACAAGATG
ANAC056-3 promoter fragment 1 reverse primer	caagaaagctgggtCGCGACACATATACAAAGCCTGACTAATC
*att*B1 Gateway forward primer	GGGGACAAGTTTGTACAAAAAAGCAGGCT
*att*B2 Gateway reverse primer	GGGGACCACTTTGTACAAGAAAGCTGGGT
M13 forward sequencing primer	GTAAAACGACGGCCAG
M13 reverse sequencing primer	CAGGAAACAGCTATGAC

Function	Sequence (5'-3')
Forward primer for creation of Y1H-235A	GGGTTCTGGTATGAATCGGTTAATTTCT
Reverse primer for creation of Y1H-235A	CAAAATAATTAACGTTTAAACCAGTCAA
Forward primer for creation of Y1H-235B	GGGAGTTCGGTTTTTGGATAACTGTTCA
Reverse primer for creation of Y1H-235B	CAAACAATTAAAGCCACGTGTGAACCTT
Forward primer for creation of Y1H-37A	CCCTAATATAATGGGATTGTAAATATAATAATTCAGTGTGAATGG
Reverse primer for creation of Y1H-37A	GAAATACGTATTAGTACCAAATTTATAACTCGAAACTACTATAAATG

Table A.3: **Primers designed for site-directed mutagenesis** Primers designed for site directed mutagenesis, with the particular mutation they were designed for. Mismatched base pairs are underlined.

B. Identification of Transcription Factors that Bind to the *ANAC092* Promoter

B.1. Final yeast 1-hybrid results for the *ANAC092* promoter

		Fragment									
ATG	Name	Fragment 1	Fragment 2	Fragment 3	Fragment 1A	Fragment 1B	Fragment 1C	Fragment 1D	Fragment 2A	Fragment 2B	Fragment 2C
AT1G25340	MYB116		3						3	3	
AT3G06490	MYB108		3						3	3	
AT1G52880	ANAC018		3						3	3	
AT1G61110	ANAC025		3						3	3	
AT3G15510	ANAC056		3						3	3	
AT5G63790	ANAC102		3						3	3	
AT1G04370	ATERF14	2			1		1	3			
AT2G36610	ATHB22						3				
AT5G65310	ATHB5						3				
AT3G23220	ESE1	3			1		1	2			
AT1G76110	HMG box protein		2								
AT1G06160	ORA59	3			1		1	3			
AT3G21175	ZML1		3						3	3	
AT2G33550	Homeodomain-like TF	3									
	3-Aminotriazole (mM)	0	20	n/a	1	1	20	1	0	0	0

Table B.1: **Final yeast 1-hybrid results for *ANAC092* promoter fragments** Table of final, pairwise yeast 1-hybrid results, with 3-aminotriazole concentrations used for each promoter fragment.

C. Additional results

Included on the CD are additional results not detailed here:

- The expression of Arabidopsis genes in *anac092-1* compared to Col 0 expressed as $\log_2$fold change (table 1).
- The expression of Arabidopsis genes differentially expressed in 35S:*ANAC056* (table 2).
- The expression of Arabidopsis genes in *anac025-1* compared to Col 0 expressed as $\log_2$fold change (table 3).
- The network file for the final yeast 1-hybrid network, including hyperparameters as determined by hCSI (table 4).
- Sequences of the NAC domains used for phylogenetic analysis used in chapter 5.
- Alignment of NAC domain sequences in clustal omega as detailed in chapter 5.

Bibliography

Abdala, G., Miersch, O., Kramell, R., Vigliocco, A., Agostini, E., Forchetti, G., & Alemano, S. (2003). Jasmonate and octadecanoid occurrence in tomato hairy roots. Endogenous level changes in response to NaCl. *Plant Growth Regulation*, 40(1), 21–27.

Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics international*, 11(7), 36–43.

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R. A., & Mengiste, T. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. *Plant Journal*, 48(1), 28–44.

Adam, Z. & Clarke, A. K. (2002). Cutting edge of chloroplast proteolysis. *Trends in Plant Science*, 7(10), 451–456.

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., & Tasaka, M. (1997). Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *The Plant cell*, 9(6), 841–857.

Äijö, T. & Lähdesmäki, H. (2009). Learning gene regulatory networks from gene expression measurements using non-parametric molecular kinetics. *Bioinformatics (Oxford, England)*, 25(22), 2937–2944.

Al-Daoud, F. & Cameron, R. K. (2011). ANAC055 and ANAC092 contribute non-redundantly in an EIN2-dependent manner to Age-Related Resistance in Arabidopsis. *Physiological and Molecular Plant Pathology*, 76(3-4), 212–222.

Albert, R. (2005). Scale-free networks in cell biology. *Journal of cell science*, 118(Pt 21), 4947–4957.

Allu, A. D., Soja, A. M., Wu, A., Szymanski, J., & Balazadeh, S. (2014). Salt stress and senescence: identification of cross-talk regulatory components. *Journal of Experimental Botany*.

Alonso, J. M. (1999). EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in Arabidopsis. *Science (New York, N.Y.)*, 284(5423), 2148–2152.

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., & Ecker, J. R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science (New York, N.Y.)*, 301(5633), 653–657.

Amasino, R. (2009). Floral induction and monocarpic versus polycarpic life histories. *Genome Biol*, 10(7), 228.

Amselem, J., Cuomo, C. A., van Kan, J. A. L., Viaud, M., Benito, E. P., Couloux, A., Coutinho, P. M., de Vries, R. P., Dyer, P. S., Fillinger, S., Fournier, E., Gout, L., Hahn, M., Kohn, L., Lapalu, N., Plummer, K. M., Pradier, J.-M., Quévillon, E., Sharon, A., Simon, A., ten Have, A., Tudzynski, B., Tudzynski, P., Wincker, P., Andrew, M., Anthouard, V., Beever, R. E., Beffa, R., Benoit, I., Bouzid, O., Brault, B., Chen, Z., Choquer, M., Collémare, J., Cotton, P., Danchin, E. G., Da Silva, C., Gautier, A., Giraud, C., Giraud, T., Gonzalez, C., Grossetete, S., Güldener, U., Henrissat, B., Howlett, B. J., Kodira, C., Kretschmer, M., Lappartient, A., Leroch, M., Levis, C., Mauceli, E., Neuvéglise, C., Oeser, B., Pearson, M., Poulain, J., Poussereau, N., Quesneville, H., Rascle, C., Schumacher, J., Ségurens, B., Sexton, A., Silva, E., Sirven, C., Soanes, D. M., Talbot, N. J., Templeton, M., Yandava, C., Yarden, O., Zeng, Q., Rollins, J. A., Lebrun, M.-H., & Dickman, M. (2011). Genomic analysis of the necrotrophic fungal pathogens Sclerotinia sclerotiorum and Botrytis cinerea. *PLoS genetics*, 7(8), e1002230.

Anderson, K. (2009). *Distortions to agricultural incentives: A global perspective, 1955-2007*. Washington DC: World Bank and Palgrave Macmillan.

Anil, K., Das, S. N., & Podile, A. R. (2013). Induced Defense in Plants: A Short Overview.

Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 84(3), 669–679.

Apel, K. & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual review of plant biology*, 55, 373–399.

Arbona, V. & Gómez-Cadenas, A. (2008). Hormonal modulation of citrus responses to flooding. *Journal of Plant Growth Regulation*, 27(3), 241–250.

Ariel, F. D., Manavella, P. A., Dezar, C. A., & Chan, R. L. (2007). The true story of the HD-Zip family. *Trends in Plant Science*, 12(9), 419–426.

Asselbergh, B., De Vleesschauwer, D., & Höfte, M. (2008). Global switches and fine-tuning-ABA modulates plant pathogen defense. *Molecular Plant-Microbe Interactions*, 21(6), 709–719.

Avila-Ospina, L., Moison, M., Yoshimoto, K., & Masclaux-Daubresse, C. (2014). Autophagy, plant senescence, and nutrient recycling. *Journal of Experimental Botany*, 65(14), 3799–3811.

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., Ren, J., Li, W. W., & Noble, W. S. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic acids research*, 37(Web Server issue), W202–8.

Bailey, T. L. & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in bipolymers.

Baker, C. C., Sieber, P., Wellmer, F., & Meyerowitz, E. M. (2005). The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. *Current biology : CB*, 15(4), 303–315.

Balazadeh, S. (2014). Stay-Green Not Always Stays Green. *Molecular Plant*.

Balazadeh, S., Kwasniewski, M., Caldana, C., Mehrnia, M., Zanor, M. I., Xue, G.-P., & Mueller-Roeber, B. (2011). ORS1, an H2O2-Responsive NAC Transcription Factor, Controls Senescence in Arabidopsis thaliana. *Molecular Plant*, 4(2), 346–360.

Balazadeh, S., Siddiqui, H., Allu, A. D., Matallana-Ramirez, L. P., Caldana, C., Mehrnia, M., Zanor, M. I., Koehler, B., & Mueller-Roeber, B. (2010a). A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. *Plant Journal*, 62(2), 250–264.

Balazadeh, S., Wu, A., & Mueller-Roeber, B. (2010b). Salt-triggered expression of the ANAC092-dependent senescence regulon in Arabidopsis thaliana. *Plant signaling & behavior*, 5(6), 733–735.

Bansal, M., Belcastro, V., Ambesi-Impiombato, A., & di Bernardo, D. (2007). How to infer gene networks from expression profiles. *Molecular systems biology*, 3, 78.

Baxter, A., Mittler, R., & Suzuki, N. (2014). ROS as key players in plant stress signalling. *Journal of Experimental Botany*, 65(5), 1229–1240.

Baxter, L., Jironkin, A., Hickman, R., Moore, J., Barrington, C., Krusche, P., Dyer, N. P., Buchanan-Wollaston, V., Tiskin, A., Beynon, J., Denby, K., & Ott, S. (2012). Conserved noncoding sequences highlight shared components of regulatory networks in dicotyledonous plants. *The Plant cell*, 24(10), 3949–3965.

Beal, M. J., Falciani, F., Ghahramani, Z., Rangel, C., & Wild, D. L. (2005). A Bayesian approach to reconstructing genetic regulatory networks with hidden factors. *Bioinformatics (Oxford, England)*, 21(3), 349–356.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, (pp. 289–300).

Berger, M. F. & Bulyk, M. L. (2009). Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nature protocols*, 4(3), 393–411.

Bilgin, D. D., Zavala, J. A., Zhu, J., Clough, S. J., Ort, D. R., & DeLUCIA, E. (2010). Biotic stress globally downregulates photosynthesis genes. *Plant Cell and Environment*, 33(10), 1597–1613.

Bilu, Y. & Barkai, N. (2005). The design of transcription-factor binding sites is affected by combinatorial regulation. *Genome biology*, 6(12), R103.

Binder, B. M., Walker, J. M., Gagne, J. M., Emborg, T. J., Hemmann, G., Bleecker, A. B., & Vierstra, R. D. (2007). The Arabidopsis EIN3 binding F-Box proteins EBF1 and EBF2 have

distinct but overlapping roles in ethylene signaling. *The Plant cell*, 19(2), 509–523.

Biswal, B. (1995). Carotenoid catabolism during leaf senescence and its control by light. *Journal of Photochemistry and Photobiology B: Biology*, 30(1), 3–13.

Blatti, C. & Sinha, S. (2014). Motif enrichment tool. *Nucleic acids research*, 42(Web Server issue), W20–5.

Bleecker, A. B. & Patterson, S. E. (1997). Last exit: senescence, abscission, and meristem arrest in Arabidopsis. *The Plant cell*, 9(7), 1169–1179.

Bolívar, J. C., Machens, F., Brill, Y., Romanov, A., Bülow, L., & Hehl, R. (2014). 'In silico expression analysis', a novel PathoPlant web tool to identify abiotic and biotic stress conditions associated with specific cis-regulatory sequences. *Database : the journal of biological databases and curation*, 2014(0), bau030.

Boller, T. & Felix, G. (2009). A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual review of plant biology*, 60(1), 379–406.

Borrell, A. K., Hammer, G. L., & Henzell, R. G. (2000). Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. *Crop Science*, 40(4), 1037–1048.

Breeze, E., Harrison, E., McHattie, S., Hughes, L., Hickman, R., Hill, C., Kiddle, S., Kim, Y.-S., Penfold, C. A., Jenkins, D., Zhang, C., Morris, K., Jenner, C., Jackson, S., Thomas, B., Tabrett, A., Legaie, R., Moore, J. D., Wild, D. L., Ott, S., Rand, D., Beynon, J., Denby, K., Mead, A., & Buchanan-Wollaston, V. (2011). High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. *The Plant cell*, 23(3), 873–894.

Broekaert, W. F., Terras, F. R., Cammue, B. P., & Osborn, R. W. (1995). Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant physiology*, 108(4), 1353–1358.

Bu, Q., Jiang, H., Li, C.-B., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q., & Li, C. (2008). Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Research*, 18(7), 756–767.

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P. O., Nam, H. G., Lin, J.-F., Wu, S.-H., Swidzinski, J., Ishizaki, K., & Leaver, C. J. (2005). Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *The Plant Journal*, 42(4), 567–585.

Bücherl, C. A., Bader, A., Westphal, A. H., Laptenok, S. P., & Borst, J. W. (2014). FRET-FLIM applications in plant systems. *Protoplasma*, 251(2), 383–394.

Calderini, O., Bovone, T., Scotti, C., Pupilli, F., Piano, E., & Arcioni, S. (2007). Delay of leaf senescence in Medicago sativa transformed with the ipt gene controlled by the senescence-specific promoter SAG12. *Plant cell reports*, 26(5), 611–615.

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M., & Powell, A. L. T. (2008). The intersection between cell wall disassembly, ripening, and fruit susceptibility to Botrytis cinerea. *Proceedings of the National Academy of Sciences of the United States of America*, 105(3), 859–864.

Cao, S., Kumimoto, R. W., Gnesutta, N., Calogero, A. M., Mantovani, R., & Holt, B. F. (2014). A Distal CCAAT/NUCLEAR FACTOR Y Complex Promotes Chromatin Looping at the FLOWERING LOCUS T Promoter and Regulates the Timing of Flowering in Arabidopsis. *The Plant cell*, 26(3), 1009–1017.

Cao, W.-H., Liu, J., He, X.-J., Mu, R.-L., Zhou, H.-L., Chen, S.-Y., & Zhang, J.-S. (2007). Modulation of ethylene responses affects plant salt-stress responses. *Plant physiology*, 143(2), 707–719.

Carrion, C. A., Costa, M. L., Martínez, D. E., Mohr, C., Humbeck, K., & Guiamet, J. J. (2013). In vivo inhibition of cysteine proteases provides evidence for the involvement of 'senescence-associated vacuoles' in chloroplast protein degradation during dark-induced senescence of tobacco leaves. *Journal of Experimental Botany*, 64(16), 4967–4980.

Carvallo, M. A., Pino, M.-T., Jeknić, Z., Zou, C., Doherty, C. J., Shiu, S.-H., Chen, T. H. H., & Thomashow, M. F. (2011). A comparison of the low temperature transcriptomes and CBF regulons of three plant species that differ in freezing tolerance: Solanum commersonii, Solanum

tuberosum, and Arabidopsis thaliana. *Journal of Experimental Botany*, 62(11), 3807–3819.

Carviel, J. L., Al-Daoud, F., Neumann, M., Mohammad, A., Provart, N. J., Moeder, W., Yoshioka, K., & Cameron, R. K. (2009). Forward and reverse genetics to identify genes involved in the age-related resistance response in Arabidopsis thaliana. *Molecular Plant Pathology*, 10(5), 621–634.

Castillo, M. C. & León, J. (2008). Expression of the beta-oxidation gene 3-ketoacyl-CoA thiolase 2 (KAT2) is required for the timely onset of natural and dark-induced leaf senescence in Arabidopsis. *Journal of Experimental Botany*, 59(8), 2171–2179.

Castrillo, G., Turck, F., Leveugle, M., Lecharny, A., Carbonero, P., Coupland, G., Paz-Ares, J., & Oñate-Sánchez, L. (2011). Speeding Cis-Trans Regulation Discovery by Phylogenomic Analyses Coupled with Screenings of an Arrayed Library of Arabidopsis Transcription Factors. *PLOS ONE*, 6(6), e21524.

Çevik, V., Kidd, B. N., Zhang, P., Hill, C., Kiddle, S., Denby, K. J., Holub, E. B., Cahill, D. M., Manners, J. M., Schenk, P. M., Beynon, J., & Kazan, K. (2012). MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in Arabidopsis. *Plant physiology*, 160(1), 541–555.

Chang, K. N., Zhong, S., Weirauch, M. T., Hon, G., Pelizzola, M., Li, H., Huang, S.-S. C., Schmitz, R. J., Urich, M. A., Kuo, D., Nery, J. R., Qiao, H., Yang, A., Jamali, A., Chen, H., Ideker, T., Ren, B., Bar-Joseph, Z., Hughes, T. R., & Ecker, J. R. (2013). Temporal transcriptional response to ethylene gas drives growth hormone cross-regulation in Arabidopsis. *eLife*, 2, e00675.

Chen, H., Hwang, J. E., Lim, C. J., Kim, D. Y., Lee, S. Y., & Lim, C. O. (2010). Arabidopsis DREB2C functions as a transcriptional activator of HsfA3 during the heat stress response. *Biochemical and biophysical research communications*, 401(2), 238–244.

Chen, H.-J., Lin, Z.-W., Huang, G.-J., & Lin, Y.-H. (2012). Sweet potato calmodulin SPCAM is involved in salt stress-mediated leaf senescence, H2O2 elevation and senescence-associated gene expression. *Journal of plant physiology*, 169(18), 1892–1902.

Chiba, A., Ishida, H., Nishizawa, N. K., Makino, A., & Mae, T. (2003). Exclusion of ribulose-1, 5-bisphosphate carboxylase/oxygenase from chloroplasts by specific bodies in naturally senescing leaves of wheat. *Plant and Cell Physiology*, 44(9), 914–921.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D. G., Felix, G., & Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, 448(7152), 497–500.

Chinnusamy, V. & Zhu, J.-K. (2009). Epigenetic regulation of stress responses in plants. *Current Opinion in Plant Biology*, 12(2), 133–139.

Cho, Y.-H., Lee, S., & Yoo, S.-D. (2012). EIN2 and EIN3 in Ethylene Signalling. In M. T. McManus (Ed.), *Annual Plant Reviews Volume 44: The Plant Hormone Ethylene* (pp. 169–187). Oxford, UK: Wiley-Blackwell.

Christiansen, M. W., Holm, P. B., & Gregersen, P. L. (2011). Characterization of barley (Hordeum vulgare L.) NAC transcription factors suggests conserved functions compared to both monocots and dicots. *BMC research notes*, 4, 302.

Christianson, J. A., Wilson, I. W., Llewellyn, D. J., & Dennis, E. S. (2009). The low-oxygen-induced NAC domain transcription factor ANAC102 affects viability of Arabidopsis seeds following low-oxygen treatment. *Plant physiology*, 149(4), 1724–1738.

Coates, M. E. & Beynon, J. L. (2010). Hyaloperonospora arabidopsidis as a pathogen model. *Annual review of phytopathology*, 48, 329–345.

Cohen, S. X., Moulin, M., Hashemolhosseini, S., Kilian, K., Wegner, M., & Müller, C. W. (2003). Structure of the GCM domain-DNA complex: a DNA-binding domain with a novel fold and mode of target site recognition. *The EMBO journal*, 22(8), 1835–1845.

Coll, N. S., Vercammen, D., Smidler, A., Clover, C., Van Breusegem, F., Dangl, J. L., & Epple, P. (2010). Arabidopsis type I metacaspases control cell death. *Science (New York, N.Y.)*, 330(6009), 1393–1397.

Creelman, R. A. & Mullet, J. E. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences*, 92(10), 4114–4119.

Cui, F., Brosché, M., Sipari, N., Tang, S., & Overmyer, K. (2013). Regulation of ABA dependent

wound induced spreading cell death by MYB108. *New Phytologist*.

Danisman, S., van Dijk, A. D. J., Bimbo, A., van der Wal, F., Hennig, L., de Folter, S., Angenent, G. C., & Immink, R. G. H. (2013). Analysis of functional redundancies within the Arabidopsis TCP transcription factor family. *Journal of Experimental Botany*, 64(18), 5673–5685.

Davies, W. J. & Zhang, J. (1991). Root signals and the regulation of growth and development of plants in drying soil. *Annual review of plant biology*, 42(1), 55–76.

De Domenico, S., Bonsegna, S., Horres, R., Pastor, V., Taurino, M., Poltronieri, P., Imtiaz, M., Kahl, G., Flors, V., Winter, P., & Santino, A. (2012). Transcriptomic analysis of oxylipin biosynthesis genes and chemical profiling reveal an early induction of jasmonates in chickpea roots under drought stress. *Plant physiology and biochemistry : PPB / Société française de physiologie végétale*, 61, 115–122.

de Torres-Zabala, M., Truman, W., Bennett, M. H., Lafforgue, G., Mansfield, J. W., Rodriguez Egea, P., Bögre, L., & Grant, M. (2007). Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *The EMBO journal*, 26(5), 1434–1443.

Debener, T., Lehnackers, H., Arnold, M., & Dangl, J. L. (1991). Identification and molecular mapping of a single Arabidopsis thaliana locus determining resistance to a phytopathogenic Pseudomonas syringae isolate. *Plant Journal*, 1(3), 289–302.

Denby, K. J., Kumar, P., & Kliebenstein, D. J. (2004). Identification of Botrytis cinerea susceptibility loci in Arabidopsis thaliana. *Plant Journal*, 38(3), 473–486.

Deplancke, B., Dupuy, D., Vidal, M., & Walhout, A. J. M. (2004). A gateway-compatible yeast one-hybrid system. *Genome research*, 14(10B), 2093–2101.

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., & Fobert, P. R. (2003). The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *The Plant cell*, 15(9), 2181–2191.

Develey-Rivière, M.-P. & Galiana, E. (2007). Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom - Develey-Rivière - 2007 - New Phytologist - Wiley Online Library. *New Phytologist*, 175(3), 405–416.

D'haeseleer, P., Liang, S., & Somogyi, R. (2000). Genetic network inference: from co-expression clustering to reverse engineering. *Bioinformatics (Oxford, England)*, 16(8), 707–726.

Díaz, J., ten Have, A., & van Kan, J. A. L. (2002). The role of ethylene and wound signaling in resistance of tomato to Botrytis cinerea. *Plant physiology*, 129(3), 1341–1351.

Dixon, D. P., Hawkins, T., Hussey, P. J., & Edwards, R. (2009). Enzyme activities and subcellular localization of members of the Arabidopsis glutathione transferase superfamily. *Journal of Experimental Botany*, 60(4), 1207–1218.

Dobi, K. C. & Winston, F. (2007). Analysis of transcriptional activation at a distance in Saccharomyces cerevisiae. *Molecular and cellular biology*, 27(15), 5575–5586.

Doelling, J. H., Walker, J. M., Friedman, E. M., Thompson, A. R., & Vierstra, R. D. (2002). The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana. *The Journal of biological chemistry*, 277(36), 33105–33114.

Dong, J., Chen, C., & Chen, Z. (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Molecular Biology*, 51(1), 21–37.

Du, J., Li, M., Kong, D., Wang, L., Lv, Q., Wang, J., Bao, F., Gong, Q., Xia, J., & He, Y. (2014). Nitric oxide induces cotyledon senescence involving co-operation of the NES1/MAD1 and EIN2-associated ORE1 signalling pathways in Arabidopsis. *Journal of Experimental Botany*, 65(14), 4051–4063.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., & Lepiniec, L. (2010). MYB transcription factors in Arabidopsis. *Trends in Plant Science*, 15(10), 573–581.

Duek, P. D. & Fankhauser, C. (2005). bHLH class transcription factors take centre stage in phytochrome signalling. *Trends in Plant Science*, 10(2), 51–54.

Duval, M., Hsieh, T.-F., Kim, S. Y., & Thomas, T. L. (2002). Molecular characterization of AtNAM: a member of the Arabidopsis NAC domain superfamily. *Plant Molecular Biology*, 50(2), 237–248.

Dwivedi, S., Kar, M., & Mishra, D. (1979). Biochemical changes in excised leaves of Oryza sativa

subjected to water stress. *Physiologia plantarum*, 45(1), 35–40.

Elad, Y. (1995). Physiological aspects of resistance to Botrytis cinerea. *Phytopathology*, 85, 637–643.

Ellis, C., Karafyllidis, I., Wasternack, C., & Turner, J. G. (2002). The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *The Plant cell*, 14(7), 1557–1566.

Ellis, C. & Turner, J. G. (2001). The Arabidopsis mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *The Plant cell*, 13(5), 1025–1033.

Ernst, H. A., Olsen, A. N., Larsen, S., & Lo Leggio, L. (2004). Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. *EMBO reports*, 5(3), 297–303.

Espino, J. J., Gutiérrez-Sánchez, G., Brito, N., Shah, P., Orlando, R., & Gonzalez, C. (2010). The Botrytis cinerea early secretome. *Proteomics*, 10(16), 3020–3034.

Estornell, L. H., Agustí, J., Merelo, P., Talón, M., & Tadeo, F. R. (2013). Elucidating mechanisms underlying organ abscission. *Plant science : an international journal of experimental plant biology*, 199-200, 48–60.

Eulgem, T. & Somssich, I. E. (2007). Networks of WRKY transcription factors in defense signaling. *Current Opinion in Plant Biology*, 10(4), 366–371.

Fan, W. & Dong, X. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. *The Plant cell*, 14(6), 1377–1389.

FAO, WFP, & IFAD (2013). The State of Food Insecurity in the World 2013.

Farage-Barhom, S., Burd, S., Sonego, L., Perl-Treves, R., & Lers, A. (2008). Expression analysis of the BFN1 nuclease gene promoter during senescence, abscission, and programmed cell death-related processes. *Journal of Experimental Botany*, 59(12), 3247–3258.

Fernández-Acero, F. J., Jorge, I., Calvo, E., Vallejo, I., Carbú, M., Camafeita, E., Garrido, C., López, J. A., Jorrin, J., & Cantoral, J. M. (2007). Proteomic analysis of phytopathogenic fungus Botrytis cinerea as a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research. *Archives of microbiology*, 187(3), 207–215.

Ferrari, S., Plotnikova, J. M., De Lorenzo, G., & Ausubel, F. M. (2003). Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *The Plant Journal*, 35(2), 193–205.

Fode, B. & Gatz, C. (2009). Chromatin immunoprecipitation experiments to investigate in vivo binding of Arabidopsis transcription factors to target sequences. *Methods in molecular biology (Clifton, N.J.)*, 479, 261–272.

Franco-Zorrilla, J. M., López-Vidriero, I., Carrasco, J. L., Godoy, M., Vera, P., & Solano, R. (2014). DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proceedings of the National Academy of Sciences of the United States of America*, 111(6), 2367–2372.

Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., Tran, L. S. P., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2004). A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *The Plant Journal*, 39(6), 863–876.

Gan, S. & Amasino, R. M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science (New York, N.Y.)*, 270(5244), 1986–1988.

Garber, R. L., Kuroiwa, A., & Gehring, W. J. (1983). Genomic and cDNA clones of the homeotic locus Antennapedia in Drosophila. *The EMBO journal*, 2(11), 2027–2036.

Geisel, N. & Gerland, U. (2011). Physical limits on cooperative protein-DNA binding and the kinetics of combinatorial transcription regulation. *Biophysical journal*, 101(7), 1569–1579.

Gepstein, S., Sabehi, G., Carp, M. J., Hajouj, T., Nesher, M. F. O., Yariv, I., Dor, C., & Bassani, M. (2003). Large-scale identification of leaf senescence-associated genes. *The Plant Journal*, 36(5), 629–642.

Ghanem, M. E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I. C., Lutts, S., & Pérez-Alfocea, F. (2008). Hormonal changes during salinity-induced leaf senescence in tomato (Solanum lycopersicum L.). *Journal of Experimental Botany*, 59(11), 3039–3050.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., & McCourt, P. (2000). Regu-

lation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. *The Plant cell*, 12(7), 1117–1126.

Gibbs, H. K., Ruesch, A. S., Achard, F., Clayton, M. K., Holmgren, P., Ramankutty, N., & Foley, J. A. (2010). Tropical forests were the primary sources of new agricultural land in the 1980s and 1990s. *Proceedings of the National Academy of Sciences of the United States of America*, 107(38), 16732–16737.

Gietz, D., St Jean, A., Woods, R. A., & Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic acids research*, 20(6), 1425.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual review of phytopathology*, 43, 205–227.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., & Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science (New York, N.Y.)*, 327(5967), 812–818.

Godoy, M., Franco-Zorrilla, J. M., Pérez-Pérez, J., Oliveros, J. C., Lorenzo, O., & Solano, R. (2011). Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. *Plant Journal*, 66(4), 700–711.

Gómez-Gómez, L. & Boller, T. (2000). FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell*, 5(6), 1003–1011.

Gong, W., Shen, Y.-P., Ma, L.-G., Pan, Y., Du, Y.-L., Wang, D.-H., Yang, J.-Y., Hu, L.-D., Liu, X.-F., Dong, C.-X., Ma, L., Chen, Y.-H., Yang, X.-Y., Gao, Y., Zhu, D., Tan, X., Mu, J.-Y., Zhang, D.-B., Liu, Y.-L., Dinesh-Kumar, S. P., Li, Y., Wang, X.-P., Gu, H.-Y., Qu, L.-J., Bai, S.-N., Lu, Y.-T., Li, J.-Y., Zhao, J.-D., Zuo, J., Huang, H., Deng, X. W., & Zhu, Y.-X. (2004). Genome-wide ORFeome cloning and analysis of Arabidopsis transcription factor genes. *Plant physiology*, 135(2), 773–782.

Govrin, E. M. & Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. *Current biology : CB*, 10(13), 751–757.

Govrin, E. M., Rachmilevitch, S., Tiwari, B. S., Solomon, M., & Levine, A. (2006). An Elicitor from Botrytis cinerea Induces the Hypersensitive Response in Arabidopsis thaliana and Other Plants and Promotes the Gray Mold Disease. *Phytopathology*, 96(3), 299–307.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology*, 29(7), 644–652.

Grant, C. E., Bailey, T. L., & Noble, W. S. (2011). FIMO: scanning for occurrences of a given motif. *Bioinformatics (Oxford, England)*, 27(7), 1017–1018.

Grbic, V. (2003). SAG2 and SAG12 protein expression in senescing Arabidopsis plants. *Physiologia plantarum*, 119(2), 263–269.

Grbic, V. & Bleecker, A. B. (1995). Ethylene regulates the timing of leaf senescence in Arabidopsis. *The Plant Journal*, 8(4), 595–602.

Gregersen, P. L., Culetic, A., Boschian, L., & Krupinska, K. (2013). Plant senescence and crop productivity. *Plant Molecular Biology*, 82(6), 603–622.

Guerineau, F., Lucy, A., & Mullineaux, P. (1992). Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. *Plant Molecular Biology*, 18(4), 815–818.

Guo, H. & Ecker, J. R. (2003). Plant Responses to Ethylene Gas Are Mediated by SCF EBF1/EBF2-Dependent Proteolysis of EIN3 Transcription Factor. *Cell*, 115(6), 667–677.

Guo, Y. & Gan, S. (2006). AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *The Plant Journal*, 46(4), 601–612.

Guo, Y. & Gan, S.-S. (2011). Convergence and divergence in gene expression profiles induced by leaf senescence and 27 senescence-promoting hormonal, pathological and environmental stress treatments. *Multiple values selected*, 35(3), 644–655.

Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L., & Noble, W. S. (2007). Quantifying similarity between motifs. *Genome Biol*, 8(2), R24.

Guzmán, P. & Ecker, J. R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *The Plant cell*, 2(6), 513–523.

Hanaoka, H., Noda, T., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., & Ohsumi, Y. (2002). Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. *Plant physiology*, 129(3), 1181–1193.

Hao, Y.-J., Song, Q.-X., Chen, H.-W., Zou, H.-F., Wei, W., Kang, X.-S., Ma, B., Zhang, W.-K., Zhang, J.-S., & Chen, S.-Y. (2010). Plant NAC-type transcription factor proteins contain a NARD domain for repression of transcriptional activation. *Planta*, 232(5), 1033–1043.

Hartley, J. L., Temple, G. F., & Brasch, M. A. (2000). DNA cloning using in vitro site-specific recombination. *Genome research*, 10(11), 1788–1795.

Have, A. t., Mulder, W., Visser, J., & van Kan, J. A. L. (1998). The Endopolygalacturonase Gene Bcpg1Is Required for Full Virulence of Botrytis cinerea. *Molecular Plant-Microbe Interactions*, 11(10), 1009–1016.

Haynes, B. C., Maier, E. J., Kramer, M. H., Wang, P. I., Brown, H., & Brent, M. R. (2013). Mapping functional transcription factor networks from gene expression data. *Genome research*, 23(8), 1319–1328.

He, X., Mu, R., Cao, W., Zhang, Z., Zhang, J., & Chen, S. (2005). AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. *Plant Journal*, 44(6), 903–916.

He, Y., Fukushige, H., Hildebrand, D. F., & Gan, S. (2002). Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. *Plant physiology*, 128(3), 876–884.

Hennig, L., Menges, M., Murray, J. A. H., & Gruissem, W. (2003). Arabidopsis transcript profiling on Affymetrix GeneChip arrays. *Plant Molecular Biology*, 53(4), 457–465.

Henriksson, E., Olsson, A. S. B., Johannesson, H., Johansson, H., Hanson, J., Engström, P., & Söderman, E. (2005). Homeodomain leucine zipper class I genes in Arabidopsis. Expression patterns and phylogenetic relationships. *Plant physiology*, 139(1), 509–518.

Hensel, L. L., Grbić, V., Baumgarten, D. A., & Bleecker, A. B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in arabidopsis. *The Plant cell*, 5(5), 553–564.

Hertz, G. Z. & Stormo, G. D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics (Oxford, England)*, 15(7-8), 563–577.

Hickman, R., Hill, C., Penfold, C. A., Breeze, E., Bowden, L., Moore, J. D., Zhang, P., Jackson, A., Cooke, E., Bewicke-Copley, F., Mead, A., Beynon, J., Wild, D. L., Denby, K. J., Ott, S., & Buchanan-Wollaston, V. (2013). A local regulatory network around three NAC transcription factors in stress responses and senescence in Arabidopsis leaves. *Plant Journal*, 75(1), 26–39.

Himelblau, E. & Amasino, R. M. (2001). Nutrients mobilized from leaves of Arabidopsis thaliana during leaf senescence. *Journal of plant physiology*, 158(10), 1317–1323.

Himmelbach, A., Hoffmann, T., Leube, M., Höhener, B., & Grill, E. (2002). Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. *The EMBO journal*, 21(12), 3029–3038.

Holz, G., Coertze, S., & Williamson, B. (2007). The ecology of Botrytis on plant surfaces. In *Botrytis: Biology, pathology and control* (pp. 9–27). Springer.

Hortensteiner, S. (2002). Nitrogen metabolism and remobilization during senescence. *Journal of Experimental Botany*, 53(370), 927–937.

Hortensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual review of plant biology*, 57, 55–77.

Hu, R., Qi, G., Kong, Y., Kong, D., Gao, Q., & Zhou, G. (2010). Comprehensive analysis of NAC domain transcription factor gene family in Populus trichocarpa. *BMC plant biology*, 10, 145.

Hughes, T. & Rusten, T. E. (2007). : (pp. 111–118). Advances in experimental medicine and biology.

Huh, S. U., Lee, S.-B., Kim, H. H., & Paek, K.-H. (2012). ATAF2, a NAC transcription factor, binds to the promoter and regulates NIT2 gene expression involved in auxin biosynthesis. *Molecules and cells*, 34(3), 305–313.

Hunková, E., Živčák, M., & Olšovská, K. (2011). Leaf area duration of oilseed rape (Brassica napus subsp. napus) varieties and hybrids and its relationship to selected growth and productivity parameters. *Journal of Central European Agriculture*, 12(1).

Huot, B., Yao, J., Montgomery, B. L., & He, S. Y. (2014). Growth-Defense Tradeoffs in Plants: A Balancing Act to Optimize Fitness. *Molecular Plant*.

Ishida, H. & Yoshimoto, K. (2008). Chloroplasts are partially mobilized to the vacuole by autophagy. *Autophagy*, 4(7), 961–962.

Ishida, H., Yoshimoto, K., Izumi, M., Reisen, D., Yano, Y., Makino, A., Ohsumi, Y., Hanson, M. R., & Mae, T. (2008). Mobilization of rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process. *Plant physiology*, 148(1), 142–155.

Jansson, S. (1994). The light-harvesting chlorophyll ab-binding proteins. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1184(1), 1–19.

Jarvis, W. R. (1977). *Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity.* Monograph, Research Branch Canada Department of Agriculture.

Jensen, M. K., Kjaersgaard, T., Nielsen, M. M., Galberg, P., Petersen, K., O'Shea, C., & Skriver, K. (2010). The Arabidopsis thaliana NAC transcription factor family: structure-function relationships and determinants of ANAC019 stress signalling. *Biochemical Journal*, 426(2), 183–196.

Jensen, M. K., Lindemose, S., Masi, F. d., Reimer, J. J., Nielsen, M., Perera, V., Workman, C. T., Turck, F., Grant, M. R., Mundy, J., Petersen, M., & Skriver, K. (2013). ATAF1 transcription factor directly regulates abscisic acid biosynthetic gene NCED3 in Arabidopsis thaliana. *FEBS Open Bio*, 3, 321–327.

Jensen, M. K. & Skriver, K. (2014). NAC transcription factor gene regulatory and protein-protein interaction networks in plant stress responses and senescence. *IUBMB life*.

Ji, Y. & Guo, H. (2013). From endoplasmic reticulum (ER) to nucleus: EIN2 bridges the gap in ethylene signaling. *Molecular Plant*, 6(1), 11–14.

Jiang, H., Li, H., Bu, Q., & Li, C. (2009). The RHA2a-interacting proteins ANAC019 and ANAC055 may play a dual role in regulating ABA response and jasmonate response. *Plant signaling & behavior*, 4(5), 464–466.

Jibran, R., A Hunter, D., & P Dijkwel, P. (2013). Hormonal regulation of leaf senescence through integration of developmental and stress signals. *Plant Molecular Biology*, 82(6), 547–561.

Jin, J., Zhang, H., Kong, L., Gao, G., & Luo, J. (2014). PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. *Nucleic acids research*, 42(Database issue), D1182–7.

Jing, H. C., Schippers, J. H. M., Hille, J., & Dijkwel, P. P. (2005). Ethylene-induced leaf senescence depends on age-related changes and OLD genes in Arabidopsis. *Journal of Experimental Botany*, 56(421), 2915–2923.

Jing, H. C., Sturre, M. J. G., Hille, J., & Dijkwel, P. P. (2002). Arabidopsis onset of leaf death-mutants identify a regulatory pathway controlling leaf senescence. *The Plant Journal*, 32(1), 51–63.

Johannesson, H., Wang, Y., & Engström, P. (2001). DNA-binding and dimerization preferences of *Arabidopsis* homeodomain-leucine zipper transcription factors *in vitro*. *Plant Molecular Biology*, 45(1), 63–73.

Johannesson, H., Wang, Y., Hanson, J., & Engström, P. (2003). The Arabidopsis thaliana homeobox gene ATHB5 is a potential regulator of abscisic acid responsiveness in developing seedlings. *Plant Molecular Biology*, 51(5), 719–729.

Jolma, A., Yan, J., Whitington, T., Toivonen, J., Nitta, K. R., Rastas, P., Morgunova, E., Enge, M., Taipale, M., Wei, G., Palin, K., Vaquerizas, J. M., Vincentelli, R., Luscombe, N. M., Hughes, T. R., Lemaire, P., Ukkonen, E., Kivioja, T., & Taipale, J. (2013). DNA-binding specificities of human transcription factors. *Cell*, 152(1-2), 327–339.

Jones, D. T., Taylor, W. R., & Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Computer applications in the biosciences : CABIOS*, 8(3), 275–282.

Jones, J. D. G. & Dangl, J. L. (2006). The plant immune system. *Nature*, 444(7117), 323–329.

Jung, C., Lyou, S. H., Yeu, S., Kim, M. A., Rhee, S., Kim, M., Lee, J. S., Choi, Y. D., & Cheong, J.-J. (2007). Microarray-based screening of jasmonate-responsive genes in Arabidopsis thaliana. *Plant cell reports*, 26(7), 1053–1063.

Kachroo, P., Shanklin, J., Shah, J., Whittle, E. J., & Klessig, D. F. (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16), 9448–9453.

Karimi, M., Inzé, D., & Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science*, 7(5), 193–195.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E., & van Kan, J. A. L. (2005). Necrotizing activity of five Botrytis cinerea endopolygalacturonases produced in Pichia pastoris. *Plant Journal*, 43(2), 213–225.

Kaufmann, K., Pajoro, A., & Angenent, G. C. (2010). Regulation of transcription in plants: mechanisms controlling developmental switches. *Nature reviews. Genetics*, 11(12), 830–842.

Keegan, L., Gill, G., & Ptashne, M. (1986). Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science (New York, N.Y.)*, 231(4739), 699–704.

Kidokoro, S., Maruyama, K., Nakashima, K., Imura, Y., Narusaka, Y., Shinwari, Z. K., Osakabe, Y., Fujita, Y., Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2009). The phytochrome-interacting factor PIF7 negatively regulates DREB1 expression under circadian control in Arabidopsis. *Plant physiology*, 151(4), 2046–2057.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., & Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant Journal*, 50(2), 347–363.

Kim, H. J., Hong, S. H., Kim, Y. W., Lee, I. H., Jun, J. H., Phee, B.-K., Rupak, T., Jeong, H., Lee, Y., Hong, B. S., Nam, H. G., Woo, H. R., & Lim, P. O. (2014). Gene regulatory cascade of senescence-associated NAC transcription factors activated by ETHYLENE-INSENSITIVE2-mediated leaf senescence signalling in Arabidopsis. *Journal of Experimental Botany*.

Kim, H. J., Ryu, H., Hong, S. H., Woo, H. R., Lim, P. O., Lee, I. C., Sheen, J., Nam, H. G., & Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(3), 814–819.

Kim, H. S., Park, B. O., Yoo, J. H., Jung, M. S., Lee, S. M., Han, H. J., Kim, K. E., Kim, S. H., Lim, C. O., Yun, D.-J., Lee, S. Y., & Chung, W. S. (2007a). Identification of a calmodulin-binding NAC protein as a transcriptional repressor in Arabidopsis. *The Journal of biological chemistry*, 282(50), 36292–36302.

Kim, H. S., Park, H. C., Kim, K. E., Jung, M. S., Han, H. J., Kim, S. H., Kwon, Y. S., Bahk, S., An, J., Bae, D. W., Yun, D.-J., Kwak, S.-S., & Chung, W. S. (2012). A NAC transcription factor and SNI1 cooperatively suppress basal pathogen resistance in Arabidopsis thaliana. *Nucleic acids research*, 40(18), 9182–9192.

Kim, J., Chung, K., & Woo, H. (2011). Three positive regulators of leaf senescence in *Arabidopsis*, ORE1, ORE3 and ORE9, play roles in crosstalk among multiple hormone-mediated senescence pathways. *Genes & Genomics*, 33, 373–381.

Kim, J. H., Woo, H. R., Kim, J., Lim, P. O., Lee, I. C., Choi, S. H., Hwang, D., & Nam, H. G. (2009). Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. *Science (New York, N.Y.)*, 323(5917), 1053–1057.

Kim, S.-G., Lee, A.-K., Yoon, H.-K., & Park, C.-M. (2008). A membrane-bound NAC transcription factor NTL8 regulates gibberellic acid-mediated salt signaling in Arabidopsis seed germination. *Plant Journal*, 55(1), 77–88.

Kim, S. Y., Kim, S.-G., Kim, Y.-S., Seo, P. J., Bae, M., Yoon, H.-K., & Park, C.-M. (2007b). Exploring membrane-associated NAC transcription factors in Arabidopsis: implications for membrane biology in genome regulation. *Nucleic acids research*, 35(1), 203–213.

Kim, Y.-S., Sakuraba, Y., Han, S.-H., Yoo, S.-C., & Paek, N.-C. (2013). Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence. *Plant and Cell Physiology*, 54(10), 1660–1672.

Kjaersgaard, T., Jensen, M. K., Christiansen, M. W., Gregersen, P., Kragelund, B. B., & Skriver,

K. (2011). Senescence-associated barley NAC (NAM, ATAF1,2, CUC) transcription factor interacts with radical-induced cell death 1 through a disordered regulatory domain. *The Journal of biological chemistry*, 286(41), 35418–35429.

Klemm, S. L. (2008). Causal Structure Identification in Nonlinear Dynamical Systems. *Master's thesis*.

Kliebenstein, D. J. & Rowe, H. C. (2008). Ecological costs of biotrophic versus necrotrophic pathogen resistance, the hypersensitive response and signal transduction. *Plant Science*, 174(6), 551–556.

Klimczak, L. J., Schindler, U., & Cashmore, A. R. (1992). DNA binding activity of the Arabidopsis G-box binding factor GBF1 is stimulated by phosphorylation by casein kinase II from broccoli. *The Plant cell*, 4(1), 87–98.

Kraepiel, Y., Pédron, J., Patrit, O., Simond-Côte, E., Hermand, V., & Van Gijsegem, F. (2011). Analysis of the plant bos1 mutant highlights necrosis as an efficient defence mechanism during D. dadantii/Arabidospis thaliana interaction. *PLOS ONE*, 6(4), e18991.

Kretschmer, M., Kassemeyer, H. H., & Hahn, M. (2007). Age-dependent Grey Mould Susceptibility and Tissue-specific Defence Gene Activation of Grapevine Berry Skins after Infection by Botrytis cinerea. *Journal of phytopathology*, 155(5), 258–263.

Kus, J. V., Zaton, K., Sarkar, R., & Cameron, R. K. (2002). Age-related resistance in Arabidopsis is a developmentally regulated defense response to Pseudomonas syringae. *The Plant cell*, 14(2), 479–490.

Laufs, P., Peaucelle, A., Morin, H., & Traas, J. (2004). MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. *Development (Cambridge, England)*, 131(17), 4311–4322.

Le, D. T., Nishiyama, R., Watanabe, Y., Mochida, K., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L. S. P. (2011). Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. *Dna Research*, 18(4), 263–276.

Lee, E. J., Matsumura, Y., Soga, K., Hoson, T., & Koizumi, N. (2007). Glycosyl Hydrolases of Cell Wall are Induced by Sugar Starvation in Arabidopsis. *Plant and Cell Physiology*, 48(3), 405–413.

Lee, S., Seo, P. J., Lee, H. J., & Park, C.-M. (2012). A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. *Plant Journal*, 70(5), 831–844.

Lee, S. C. & Luan, S. (2012). ABA signal transduction at the crossroad of biotic and abiotic stress responses. *Plant Cell and Environment*, 35(1), 53–60.

Lee, T.-M., Lur, H.-S., & Chu, C. (1997). Role of abscisic acid in chilling tolerance of rice (Oryza sativa L.) seedlings.: II. Modulation of free polyamine levels. *Plant Science*, 126(1), 1–10.

Lehmann, J., Atzorn, R., Brückner, C., Reinbothe, S., Leopold, J., Wasternack, C., & Parthier, B. (1995). Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments. *Planta*, 197(1), 156–162.

Lei, G., Shen, M., Li, Z.-G., Zhang, B., Duan, K.-X., Wang, N., Cao, Y.-R., Zhang, W.-K., Ma, B., Ling, H.-Q., Chen, S.-Y., & Zhang, J.-S. (2011). EIN2 regulates salt stress response and interacts with a MA3 domain-containing protein ECIP1 in Arabidopsis. *Plant Cell and Environment*, 34(10), 1678–1692.

Leivar, P., Monte, E., Al-Sady, B., Carle, C., Storer, A., Alonso, J. M., Ecker, J. R., & Quail, P. H. (2008). The Arabidopsis phytochrome-interacting factor PIF7, together with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB levels. *The Plant cell*, 20(2), 337–352.

Li, J., Brader, G., Kariola, T., & Tapio Palva, E. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *The Plant Journal*, 46(3), 477–491.

Li, J., Brader, G., & Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *The Plant cell*, 16(2), 319–331.

Li, J. J. & Herskowitz, I. (1993). Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science (New York, N.Y.)*, 262(5141), 1870–1874.

Li, L., Ljung, K., Breton, G., Schmitz, R. J., Pruneda-Paz, J., Cowing-Zitron, C., Cole, B. J., Ivans, L. J., Pedmale, U. V., Jung, H.-S., Ecker, J. R., Kay, S. A., & Chory, J. (2012). Linking photoreceptor excitation to changes in plant architecture. *Genes & Development*, 26(8), 785–790.

Li, Z., Peng, J., Wen, X., & Guo, H. (2013). Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. *THE PLANT CELL ONLINE*, 25(9), 3311–3328.

Licausi, F., Ohme-Takagi, M., & Perata, P. (2013). APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytologist*, 199(3), 639–649.

Lim, P. O., Kim, H. J., & Nam, H. G. (2007). Leaf senescence. *Annual review of plant biology*, 58, 115–136.

Lin, J.-F. & Wu, S.-H. (2004). Molecular events in senescing Arabidopsis leaves. *Plant Journal*, 39(4), 612–628.

Lin, Z., Hong, Y., Yin, M., Li, C., Zhang, K., & Grierson, D. (2008). A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *The Plant Journal*, 55(2), 301–310.

Lindemose, S., Jensen, M. K., de Velde, J. V., O'Shea, C., Heyndrickx, K. S., Workman, C. T., Vandepoele, K., Skriver, K., & Masi, F. d. (2014). A DNA-binding-site landscape and regulatory network analysis for NAC transcription factors in Arabidopsis thaliana. *Nucleic acids research*.

Lindemose, S., O'Shea, C., Jensen, M. K., & Skriver, K. (2013). Structure, function and networks of transcription factors involved in abiotic stress responses. *International journal of molecular sciences*, 14(3), 5842–5878.

Liu, L., Zhou, Y., Szczerba, M. W., Li, X., & Lin, Y. (2010). Identification and application of a rice senescence-associated promoter. *Plant physiology*, 153(3), 1239–1249.

Lloyd, A. J., William Allwood, J., Winder, C. L., Dunn, W. B., Heald, J. K., Cristescu, S. M., Sivakumaran, A., Harren, F. J. M., Mulema, J., Denby, K., Goodacre, R., Smith, A. R., & Mur, L. A. J. (2011). Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against Botrytis cinerea. *Plant Journal*, 67(5), 852–868.

Lohman, K. N., Gan, S., John, M. C., & Amasino, R. M. (1994). Molecular analysis of natural leaf senescence in Arabidopsis thaliana. *Physiologia plantarum*, 92(2), 322–328.

Lopez-Molina, L., Mongrand, S., & Chua, N.-H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proceedings of the National Academy of Sciences*, 98(8), 4782–4787.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., & Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant cell*, 15(1), 165–178.

Lu, P.-L., Chen, N.-Z., An, R., Su, Z., Qi, B.-S., Ren, F., Chen, J., & Wang, X.-C. (2006). A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. *Plant Molecular Biology*, 63(2), 289–305.

Maere, S., Heymans, K., & Kuiper, M. (2005). BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics (Oxford, England)*, 21(16), 3448–3449.

Makino, A., Mae, T., & Ohira, K. (1984). Relation between nitrogen and ribulose-1, 5-bisphosphate carboxylase in rice leaves from emergence through senescence. *Plant and Cell Physiology*, 25(3), 429–437.

Mallappa, C., Singh, A., Ram, H., & Chattopadhyay, S. (2008). GBF1, a Transcription Factor of Blue Light Signaling in Arabidopsis, Is Degraded in the Dark by a Proteasome-mediated Pathway Independent of COP1 and SPA1. *Journal of Biological Chemistry*, 283(51), 35772–35782.

Mallappa, C., Yadav, V., Negi, P., & Chattopadhyay, S. (2006). A basic leucine zipper transcription factor, G-box-binding factor 1, regulates blue light-mediated photomorphogenic growth in Arabidopsis. *The Journal of biological chemistry*, 281(31), 22190–22199.

Mallory, A. C., Dugas, D. V., Bartel, D. P., & Bartel, B. (2004). MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Current biology : CB*, 14(12), 1035–1046.

Manavella, P. A., Dezar, C. A., & Bonaventure, G. (2008). HAHB4, a sunflower HD-Zip protein, integrates signals from the jasmonic acid and ethylene pathways during wounding and biotic stress responses - Manavella - 2008 - The Plant Journal - Wiley Online Library. *The Plant ...*.

Mandaokar, A. & Browse, J. (2009). MYB108 acts together with MYB24 to regulate jasmonate-mediated stamen maturation in Arabidopsis. *Plant physiology*, 149(2), 851–862.

Mangan, S. & Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proceedings of the National Academy of Sciences*, 100(21), 11980–11985.

Manteau, S., Abouna, S., Lambert, B., & Legendre, L. (2003). Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus Botrytis cinerea. *FEMS Microbiology Ecology*, 43(3), 359–366.

Marchler-Bauer, A. & Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. *Nucleic acids research*, 32(Web Server issue), W327–31.

Marioni, J. C., Mason, C. E., Mane, S. M., Stephens, M., & Gilad, Y. (2008). RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. *Genome research*, 18(9), 1509–1517.

Martínez, D. E., Costa, M. L., Gomez, F. M., Otegui, M. S., & Guiamet, J. J. (2008). 'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves. *Plant Journal*, 56(2), 196–206.

Martinez, N. J., Ow, M. C., Barrasa, M. I., Hammell, M., Sequerra, R., Doucette-Stamm, L., Roth, F. P., Ambros, V. R., & Walhout, A. J. M. (2008). A C. elegans genome-scale microRNA network contains composite feedback motifs with high flux capacity. *Genes & Development*, 22(18), 2535–2549.

Masclaux-Daubresse, C. & Chardon, F. (2011). Exploring nitrogen remobilization for seed filling using natural variation in Arabidopsis thaliana. *Journal of Experimental Botany*, 62(6), 2131–2142.

Masclaux-Daubresse, C., Reisdorf-Cren, M., & Orsel, M. (2008). Leaf nitrogen remobilisation for plant development and grain filling. *Plant biology (Stuttgart, Germany)*, 10 Suppl 1, 23–36.

Maskos, U. & Southern, E. M. (1992). Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ. *Nucleic acids research*, 20(7), 1679–1684.

Matallana-Ramirez, L. P., Rauf, M., Farage-Barhom, S., Dortay, H., Xue, G.-P., Dröge-Laser, W., Lers, A., Balazadeh, S., & Mueller-Roeber, B. (2013). NAC Transcription Factor ORE1 and Senescence-Induced BIFUNCTIONAL NUCLEASE1 (BFN1) Constitute a Regulatory Cascade in Arabidopsis. *Molecular Plant*, 6(5), 1432–1452.

Mathelier, A., Zhao, X., Zhang, A. W., Parcy, F., Worsley-Hunt, R., Arenillas, D. J., Buchman, S., Chen, C.-y., Chou, A., Ienasescu, H., Lim, J., Shyr, C., Tan, G., Zhou, M., Lenhard, B., Sandelin, A., & Wasserman, W. W. (2014). JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic acids research*, 42(Database issue), D142–7.

Mayda, E., Tornero, P., Conejero, V., & Vera, P. (1999). A tomato homeobox gene (HD-zip) is involved in limiting the spread of programmed cell death. *Plant Journal*, 20(5), 591–600.

McCabe, M. S., Garratt, L. C., Schepers, F., Jordi, W. J., Stoopen, G. M., Davelaar, E., van Rhijn, J. H. A., Power, J. B., & Davey, M. R. (2001). Effects of PSAG12-IPT gene expression on development and senescence in transgenic lettuce. *Plant physiology*, 127(2), 505–516.

McElver, J., Tzafrir, I., Aux, G., Rogers, R., Ashby, C., Smith, K., Thomas, C., Schetter, A., Zhou, Q., Cushman, M. A., Tossberg, J., Nickle, T., Levin, J. Z., Law, M., Meinke, D., & Patton, D. (2001). Insertional mutagenesis of genes required for seed development in Arabidopsis thaliana. *Genetics*, 159(4), 1751–1763.

McHattie, S. (2011). *Modelling transcriptional networks in plant senescence*. PhD thesis, University of Warwick.

Meijer, A. H., de Kam, R. J., d'Erfurth, I., Shen, W., & Hoge, J. H. C. (2000). HD-Zip proteins of families I and II from rice: interactions and functional properties. *MGG - Molecular and General Genetics*, 263(1), 12–21.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., & He, S. Y. (2006). Plant stomata function

in innate immunity against bacterial invasion. *Cell*, 126(5), 969–980.

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rohde, A., & Beeckman, T. (2008). Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. *Nature Genetics*, 40(12), 1489–1492.

Mengiste, T., Chen, X., Salmeron, J., & Dietrich, R. (2003). The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. *The Plant cell*, 15(11), 2551–2565.

Merewitz, E. B., Du, H., Yu, W., Liu, Y., Gianfagna, T., & Huang, B. (2012). Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. *Journal of Experimental Botany*, 63(3), 1315–1328.

Merewitz, E. B., Gianfagna, T., & Huang, B. (2011). Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. *Journal of Experimental Botany*, 62(15), 5311–5333.

Meyerowitz, E. M. (2002). Plants compared to animals: the broadest comparative study of development. *Science (New York, N.Y.)*, 295(5559), 1482–1485.

Mi, J. K., Mi-Jeong, P., Pil, J. S., Jin-Su, S., Hie-Joon, K., & Chung-Mo, P. (2012). Controlled nuclear import of the transcription factor NTL6 reveals a cytoplasmic role of SnRK2. 8 in the drought-stress response. *Biochemical Journal*, 448(3), 353–363.

Miao, Y., Laun, T., Zimmermann, P., & Zentgraf, U. (2004). Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. *Plant Molecular Biology*, 55(6), 853–867.

Mirzaei, H., Knijnenburg, T. A., Kim, B., Robinson, M., Picotti, P., Carter, G. W., Li, S., Dilworth, D. J., Eng, J. K., Aitchison, J. D., Shmulevich, I., Galitski, T., Aebersold, R., & Ranish, J. (2013). Systematic measurement of transcription factor-DNA interactions by targeted mass spectrometry identifies candidate gene regulatory proteins. *Proc Natl Acad Sci USA*, 110(9), 3645–3650.

Mishra, S., Shukla, A., Upadhyay, S., Sanchita, Sharma, P., Singh, S., Phukan, U. J., Meena, A., Khan, F., Tripathi, V., Shukla, R. K., & Shrama, A. (2014). Identification, occurrence, and validation of DRE and ABRE Cis-regulatory motifs in the promoter regions of genes of Arabidopsis thaliana. *Journal of Integrative Plant Biology*, 56(4), 388–399.

Mitsuda, N., Hisabori, T., Takeyasu, K., & Sato, M. H. (2004). VOZ; isolation and characterization of novel vascular plant transcription factors with a one-zinc finger from Arabidopsis thaliana. *Plant and Cell Physiology*, 45(7), 845–854.

Mitsuda, N., Ikeda, M., Takada, S., Takiguchi, Y., Kondou, Y., Yoshizumi, T., Fujita, M., Shinozaki, K., Matsui, M., & Ohme-Takagi, M. (2010). *Efficient yeast one-/two-hybrid screening using a library composed only of transcription factors in Arabidopsis thaliana.* Technical report, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, Higashi 1-1-1, Tsukuba, 305-8562 Japan. nobutaka.mitsuda@aist.go.jp.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., & Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 104(49), 19613–19618.

Molina, C. & Grotewold, E. (2005). Genome wide analysis of Arabidopsis core promoters. *BMC genomics*, 6, 25.

Monte, E., Alonso, J. M., Ecker, J. R., Zhang, Y., Li, X., Young, J., Austin-Phillips, S., & Quail, P. H. (2003). Isolation and characterization of phyC mutants in Arabidopsis reveals complex crosstalk between phytochrome signaling pathways. *The Plant cell*, 15(9), 1962–1980.

Moreno-Risueno, M. A., Busch, W., & Benfey, P. N. (2010). Omics meet networks - using systems approaches to infer regulatory networks in plants. *Current Opinion in Plant Biology*, 13(2), 126–131.

Morris, K., MacKerness, S. A., Page, T., John, C. F., Murphy, A. M., Carr, J. P., & Buchanan-Wollaston, V. (2000). Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant Journal*, 23(5), 677–685.

Munné-Bosch, S. & Alegre, L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. *Functional Plant Biology*, 31(3), 203–216.

Murmu, J., Wilton, M., Allard, G., Pandeya, R., Desveaux, D., Singh, J., & Subramaniam, R.

(2013). Arabidopsis Golden2-like transcription factors (GLK) activate JA-dependent disease susceptibility against the biotrophic pathogen Hyaloperonospora arabidopsidisas well as JA-independent plant immunity against the necrotrophic pathogen Botrytis cinerea. *Molecular Plant Pathology*, (pp. n/a–n/a).

Murphy, K. & Mian, S. (1999). Modelling gene expression data using dynamic Bayesian networks. (pp. 1–12).

Nakamura, S., Mano, S., Tanaka, Y., Ohnishi, M., Nakamori, C., Araki, M., Niwa, T., Nishimura, M., Kaminaka, H., & Nakagawa, T. (2010). Gateway binary vectors with the bialaphos resistance gene, bar, as a selection marker for plant transformation. *Bioscience, biotechnology, and biochemistry*, 74(6), 1315–1319.

Nakano, T., Suzuki, K., Fujimura, T., & Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant physiology*, 140(2), 411–432.

Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). NAC transcription factors in plant abiotic stress responses. *Biochimica et biophysica acta*, 1819(2), 97–103.

Nambara, E., Okamoto, M., Tatematsu, K., Yano, R., Seo, M., & Kamiya, Y. (2010). Abscisic acid and the control of seed dormancy and germination. *Seed Science Research*, 20(2), 55.

Navabpour, S., Morris, K., Allen, R., Harrison, E., A-H-Mackerness, S., & Buchanan-Wollaston, V. (2003). Expression of senescence-enhanced genes in response to oxidative stress. *Journal of Experimental Botany*, 54(391), 2285–2292.

Ndamukong, I., Abdallat, A. A., Thurow, C., Fode, B., Zander, M., Weigel, R., & Gatz, C. (2007). SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. *The Plant Journal*, 50(1), 128–139.

Nimchuk, Z., Eulgem, T., Holt Iii, B. F., & Dangl, J. L. (2003). Recognition and response in the plant immune system. *Annual review of genetics*, 37(1), 579–609.

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D. T., Kojima, M., Werner, T., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K., Kakimoto, T., Sakakibara, H., Schmülling, T., & Tran, L. S. P. (2011). Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *THE PLANT CELL ONLINE*, 23(6), 2169–2183.

Noh, Y.-S. & Amasino, R. M. (1999). Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Molecular Biology*, 41(2), 181–194.

Nuruzzaman, M., Manimekalai, R., Sharoni, A. M., Satoh, K., Kondoh, H., Ooka, H., & Kikuchi, S. (2010). Genome-wide analysis of NAC transcription factor family in rice. *Gene*, 465(1-2), 30–44.

Oh, S. A., Park, J. H., Lee, G. I., Paek, K. H., Park, S. K., & Nam, H. G. (1997). Identification of three genetic loci controlling leaf senescence in Arabidopsis thaliana. *Plant Journal*, 12(3), 527–535.

Oliver, R. P. & Ipcho, S. V. S. (2004). Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. *Molecular Plant Pathology*, 5(4), 347–352.

Olsen, A. N., Ernst, H. A., Leggio, L. L., & Skriver, K. (2005a). DNA-binding specificity and molecular functions of NAC transcription factors. *Plant Science*, 169(4), 785–797.

Olsen, A. N., Ernst, H. A., Leggio, L. L., & Skriver, K. (2005b). NAC transcription factors: structurally distinct, functionally diverse. *Trends in Plant Science*, 10(2), 79–87.

Olsen, A. N., Ernst, H. A., Lo Leggio, L., Johansson, E., Larsen, S., & Skriver, K. (2003). Preliminary crystallographic analysis of the NAC domain of ANAC, a member of the plant-specific NAC transcription factor family. *Acta Crystallographica Section D Biological Crystallography*, 60(1), 112–115.

Olsson, A. S. B., Engström, P., & Söderman, E. (2004). The homeobox genes ATHB12 and ATHB7 encode potential regulators of growth in response to water deficit in Arabidopsis. *Plant Molecular Biology*, 55(5), 663–677.

Oñate-Sánchez, L., Anderson, J. P., Young, J., & Singh, K. B. (2007). AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant physiology*, 143(1), 400–409.

Oñate-Sánchez, L. & Singh, K. B. (2002). Identification of Arabidopsis ethylene-responsive element

binding factors with distinct induction kinetics after pathogen infection. *Plant physiology*, 128(4), 1313–1322.

Ooka, H., Satoh, K., Doi, K., Nagata, T., Otomo, Y., Murakami, K., Matsubara, K., Osato, N., Kawai, J., Carninci, P., Hayashizaki, Y., Suzuki, K., Kojima, K., Takahara, Y., Yamamoto, K., & Kikuchi, S. (2003). Comprehensive analysis of NAC family genes in Oryza sativa and Arabidopsis thaliana. *Dna Research*, 10(6), 239–247.

Osborne, D. J. (1962). Effect of kinetin on protein & nucleic acid metabolism in Xanthium leaves during senescence. *Plant physiology*, 37(5), 595.

Otegui, M. S., Noh, Y.-S., Martínez, D. E., Vila Petroff, M. G., Staehelin, L. A., Amasino, R. M., & Guiamet, J. J. (2005). Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. *Plant Journal*, 41(6), 831–844.

Ou, B., Yin, K.-Q., Liu, S.-N., Yang, Y., Gu, T., Wing Hui, J. M., Zhang, L., Miao, J., Kondou, Y., Matsui, M., Gu, H.-Y., & Qu, L.-J. (2011). A high-throughput screening system for Arabidopsis transcription factors and its application to Med25-dependent transcriptional regulation. *Molecular Plant*, 4(3), 546–555.

Ougham, H. J., Morris, P., & Thomas, H. (2005). The colors of autumn leaves as symptoms of cellular recycling and defenses against environmental stresses. *Current topics in developmental biology*, 66, 135–160.

Panstruga, R. (2003). Establishing compatibility between plants and obligate biotrophic pathogens. *Current Opinion in Plant Biology*, 6(4), 320–326.

Park, J.-H., Oh, S. A., Kim, Y. H., Woo, H. R., & Nam, H. G. (1998). Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. *Plant Molecular Biology*, 37(3), 445–454.

Paz-Ares, J. & Regia Consortium (2002). REGIA, an EU project on functional genomics of transcription factors from Arabidopsis Thaliana. *Comparative and functional genomics*, 3(2), 102–108.

Peaucelle, A., Morin, H., Traas, J., & Laufs, P. (2007). Plants expressing a miR164-resistant CUC2 gene reveal the importance of post-meristematic maintenance of phyllotaxy in Arabidopsis. *Development (Cambridge, England)*, 134(6), 1045–1050.

Penfold, C. A., Buchanan-Wollaston, V., Denby, K. J., & Wild, D. L. (2012). Nonparametric Bayesian inference for perturbed and orthologous gene regulatory networks. *Bioinformatics (Oxford, England)*, 28(12), i233–i241.

Penfold, C. A. & Wild, D. L. (2011). How to infer gene networks from expression profiles, revisited. *Interface Focus*, 1(6), 857–870.

Penninckx, I. A., Thomma, B. P., Buchala, A., Métraux, J. P., & Broekaert, W. F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *The Plant cell*, 10(12), 2103–2113.

Pérez-Amador, M. A., Abler, M. L., De Rocher, E. J., Thompson, D. M., van Hoof, A., LeBrasseur, N. D., Lers, A., & Green, P. J. (2000). Identification of BFN1, a bifunctional nuclease induced during leaf and stem senescence in Arabidopsis. *Plant physiology*, 122(1), 169–180.

Pérez-Pérez, J. M., Esteve-Bruna, D., González-Bayón, R., Kangasjärvi, S., Caldana, C., Hannah, M. A., Willmitzer, L., Ponce, M. R., & Micol, J. L. (2013). Functional Redundancy and Divergence within the Arabidopsis RETICULATA-RELATED Gene Family. *Plant physiology*, 162(2), 589–603.

Phillips, A. R., Suttangkakul, A., & Vierstra, R. D. (2008). The ATG12-conjugating enzyme ATG10 Is essential for autophagic vesicle formation in Arabidopsis thaliana. *Genetics*, 178(3), 1339–1353.

Pianelli, K., Mari, S., Marquès, L., Lebrun, M., & Czernic, P. (2005). Nicotianamine overaccumulation confers resistance to nickel in Arabidopsis thaliana. *Transgenic research*, 14(5), 739–748.

Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annual review of cell and developmental biology*, 28, 489–521.

Pospíšilová, J. & Baťková, P. (2004). Effects of pre-treatments with abscisic acid and/or benzyladenine on gas exchange of French bean, sugar beet, and maize leaves during water stress and after

rehydration. *Biologia Plantarum*, 48(3), 395–399.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., & Genschik, P. (2003). EIN3-Dependent Regulation of Plant Ethylene Hormone Signaling by Two Arabidopsis F Box Proteins: EBF1 and EBF2. *Cell*, 115(6), 679–689.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C. M. J., & Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant physiology*, 147(3), 1347–1357.

Pruneda-Paz, J. L., Breton, G., Nagel, D. H., Kang, S. E., Bonaldi, K., Doherty, C. J., Ravelo, S., Galli, M., Ecker, J. R., & Kay, S. A. (2014). A Genome-Scale Resource for the Functional Characterization of Arabidopsis Transcription Factors. *Cell reports*.

Pruneda-Paz, J. L., Breton, G., Para, A., & Kay, S. A. (2009). A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. *Science (New York, N.Y.)*, 323(5920), 1481–1485.

Puranik, S., Sahu, P. P., Srivastava, P. S., & Prasad, M. (2012). NAC proteins: regulation and role in stress tolerance. *Trends in Plant Science*, 17(6), 369–381.

Qadir, A., Hewett, E. W., & Long, P. G. (1997). Ethylene production by Botrytis cinerea. *Postharvest Biology and Technology*, 11(2), 85–91.

Quirino, B. F., Normanly, J., & Amasino, R. M. (1999). Diverse range of gene activity during Arabidopsis thaliana leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Molecular Biology*, 40(2), 267–278.

Raman, S., Greb, T., Peaucelle, A., Blein, T., Laufs, P., & Theres, K. (2008). Interplay of miR164, CUP-SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in Arabidopsis thaliana. *Plant Journal*, 55(1), 65–76.

Ramankutty, N., Evan, A. T., Monfreda, C., & Foley, J. A. (2008). Farming the planet: 1. Geographic distribution of global agricultural lands in the year 2000. *Global Biogeochemical Cycles*, 22(1).

Rauf, M., Arif, M., Dortay, H., Matallana-Ramirez, L. P., Waters, M. T., Gil Nam, H., Lim, P. O., Mueller-Roeber, B., & Balazadeh, S. (2013). ORE1 balances leaf senescence against maintenance by antagonizing G2-like-mediated transcription. *EMBO reports*, 14(4), 382–388.

Reino, J. L., Hernández Galán, R., Durán Patrón, R., & Collado, I. G. (2004). Virulence–toxin production relationship in isolates of the plant pathogenic fungus Botrytis cinerea. *Journal of phytopathology*, 152(10), 563–566.

Rensing, S. A., Lang, D., Zimmer, A. D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.-F., Lindquist, E. A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S.-I., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W. B., Barker, E., Bennetzen, J. L., Blankenship, R., Cho, S. H., Dutcher, S. K., Estelle, M., Fawcett, J. A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K. A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D. R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P. J., Sanderfoot, A., Schween, G., Shiu, S.-H., Stueber, K., Theodoulou, F. L., Tu, H., Van de Peer, Y., Verrier, P. J., Waters, E., Wood, A., Yang, L., Cove, D., Cuming, A. C., Hasebe, M., Lucas, S., Mishler, B. D., Reski, R., Grigoriev, I. V., Quatrano, R. S., & Boore, J. L. (2008). The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. *Science (New York, N.Y.)*, 319(5859), 64–69.

Revyakin, A., Zhang, Z., Coleman, R. A., Li, Y., Inouye, C., Lucas, J. K., Park, S.-R., Chu, S., & Tjian, R. (2012). Transcription initiation by human RNA polymerase II visualized at single-molecule resolution. *Genes & Development*, 26(15), 1691–1702.

Richmon, A. E. & Land, A. (1957). Effect of Kinetin on Protein Content and Survival of Detached Xanthium Leaves. *Science (New York, N.Y.)*, 125(3249), 650–651.

Riechmann, J. L. (2002). Transcriptional Regulation: a Genomic Overview. *The Arabidopsis Book*, 16(1), 1.

Riechmann, J. L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O. J., Samaha, R. R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J. Z., Ghandehari, D., Sherman, B. K., & Yu, G. (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science (New York, N.Y.)*, 290(5499), 2105–2110.

Rivas-San Vicente, M. & Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany*, 62(10), 3321–3338.

Rivero, R. M., Gimeno, J., Van Deynze, A., Walia, H., & Blumwald, E. (2010). Enhanced cytokinin synthesis in tobacco plants expressing PSARK::IPT prevents the degradation of photosynthetic protein complexes during drought. *Plant and Cell Physiology*, 51(11), 1929–1941.

Rivero, R. M., Kojima, M., Gepstein, A., Sakakibara, H., Mittler, R., Gepstein, S., & Blumwald, E. (2007). Delayed leaf senescence induces extreme drought tolerance in a flowering plant. *Proceedings of the National Academy of Sciences of the United States of America*, 104(49), 19631–19636.

Roberts, I. N., Caputo, C., Criado, M. V., & Funk, C. (2012). Senescence-associated proteases in plants. *Physiologia plantarum*, 145(1), 130–139.

Robson, P. R., Donnison, I. S., Wang, K., Frame, B., Pegg, S. E., Thomas, A., & Thomas, H. (2004). Leaf senescence is delayed in maize expressing the Agrobacterium IPT gene under the control of a novel maize senescence-enhanced promoter. *Plant Biotechnology Journal*, 2(2), 101–112.

Romero, I., Fuertes, A., Benito, M. J., Malpica, J. M., Leyva, A., & Paz-Ares, J. (1998). More than 80R2R3-MYB regulatory genes in the genome of Arabidopsis thaliana. *Plant Journal*, 14(3), 273–284.

Ruberti, I., Sessa, G., Lucchetti, S., & Morelli, G. (1991). A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. *The EMBO journal*, 10(7), 1787–1791.

Rusterucci, C., Zhao, Z., Haines, K., Mellersh, D., Neumann, M., & Cameron, R. K. (2005). Age-related resistance to Pseudomonas syringae pv. tomato is associated with the transition to flowering in Arabidopsis and is effective against Peronospora parasitica. *Physiological and Molecular Plant Pathology*, 66(6), 222–231.

Sakuraba, Y., Park, S.-Y., Kim, Y.-S., Wang, S.-H., Yoo, S.-C., Hörtensteiner, S., & Paek, N.-C. (2014). Arabidopsis STAY-GREEN2 Is a Negative Regulator of Chlorophyll Degradation during Leaf Senescence. *Molecular Plant*.

Sakuraba, Y., Schelbert, S., Park, S.-Y., Han, S.-H., Lee, B.-D., Andrès, C. B., Kessler, F., Hörtensteiner, S., & Paek, N.-C. (2012). STAY-GREEN and chlorophyll catabolic enzymes interact at light-harvesting complex II for chlorophyll detoxification during leaf senescence in Arabidopsis. *The Plant cell*, 24(2), 507–518.

Schena, M. & Davis, R. W. (1992). HD-Zip proteins: members of an Arabidopsis homeodomain protein superfamily. *Proceedings of the National Academy of Sciences of the United States of America*, 89(9), 3894–3898.

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science (New York, N.Y.)*, 270(5235), 467–470.

Schindler, U., Menkens, A. E., Beckmann, H., Ecker, J. R., & Cashmore, A. R. (1992a). Heterodimerization between light-regulated and ubiquitously expressed Arabidopsis GBF bZIP proteins. *The EMBO journal*, 11(4), 1261–1273.

Schindler, U., Terzaghi, W. B., Beckmann, H., Kadesch, T., & Cashmore, A. R. (1992b). DNA binding site preferences and transcriptional activation properties of the Arabidopsis transcription factor GBF1. *The EMBO journal*, 11(4), 1275–1289.

Schommer, C., Palatnik, J. F., Aggarwal, P., Chételat, A., Cubas, P., Farmer, E. E., Nath, U., & Weigel, D. (2008). Control of jasmonate biosynthesis and senescence by miR319 targets. *PLOS Biology*, 6(9), e230.

Schouten, A., Tenberge, K. B., Vermeer, J., Stewart, J., Wagemakers, L., Williamson, B., & van Kan, J. A. (2002). Functional analysis of an extracellular catalase of Botrytis cinerea. *Molecular Plant Pathology*, 3(4), 227–238.

Schumacher, J. & Tudzynski, P. (2012). Morphogenesis and infection in Botrytis cinerea. In *Morphogenesis and Pathogenicity in Fungi* (pp. 225–241). Berlin: Springer.

Sclep, G., Allemeersch, J., Liechti, R., De Meyer, B., Beynon, J., Bhalerao, R., Moreau, Y., Nietfeld, W., Renou, J.-P., Reymond, P., Kuiper, M. T., & Hilson, P. (2007). CATMA, a comprehensive genome-scale resource for silencing and transcript profiling of Arabidopsis genes. *BMC Bioinformatics*, 8(1), 400.

Seltmann, M. A., Hussels, W., & Berger, S. (2010a). Jasmonates during senescence. *Plant signaling*

& behavior, 5(11), 1493–1496.

Seltmann, M. A., Stingl, N. E., Lautenschlaeger, J. K., Krischke, M., Mueller, M. J., & Berger, S. (2010b). Differential impact of lipoxygenase 2 and jasmonates on natural and stress-induced senescence in Arabidopsis. *Plant physiology*, 152(4), 1940–1950.

Seo, P. J., Kim, M. J., Park, J. Y., Kim, S. Y., Jeon, J., Lee, Y. H., Kim, J., & Park, C.-M. (2010). Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. *Plant Journal*, 61(4), 661–671.

Seo, P. J., Park, J.-M., Kang, S. K., Kim, S.-G., & Park, C.-M. (2011). An Arabidopsis senescence-associated protein SAG29 regulates cell viability under high salinity. *Planta*, 233(1), 189–200.

Sessa, G., Morelli, G., & Ruberti, I. (1993). The Athb-1 and -2 HD-Zip Domains Homodimerize forming complexes of different DNA binding specificities. *The EMBO journal*, 12, 3507–3517.

Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J. D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F., Glazebrook, J., Law, M., & Goff, S. A. (2002). A high-throughput Arabidopsis reverse genetics system. *The Plant cell*, 14(12), 2985–2994.

Shahnejat-Bushehri, S., Mueller-Roeber, B., & Balazadeh, S. (2012). Arabidopsis NAC transcription factor JUNGBRUNNEN1 affects thermomemory-associated genes and enhances heat stress tolerance in primed and unprimed conditions. *Plant signaling & behavior*, 7(12), 1518–1521.

Shi, Y., An, L., Li, X., Huang, C., & Chen, G. (2011). The octadecanoid signaling pathway participates in the chilling-induced transcription of ω-3 fatty acid desaturases in Arabidopsis. *Plant physiology and biochemistry : PPB / Société française de physiologie végétale*, 49(2), 208–215.

Shiu, S.-H. & Bleecker, A. B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. *Plant physiology*, 132(2), 530–543.

Shiu, S.-H., Shih, M.-C., & Li, W.-H. (2005). Transcription Factor Families Have Much Higher Expansion Rates in Plants than in Animals. *Plant physiology*, 139(1), 18–26.

Sieber, P., Wellmer, F., Gheyselinck, J., Riechmann, J. L., & Meyerowitz, E. M. (2007). Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development (Cambridge, England)*, 134(6), 1051–1060.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology*, 7, 539.

Siewers, V., Kokkelink, L., Smedsgaard, J., & Tudzynski, P. (2006). Identification of an abscisic acid gene cluster in the grey mold Botrytis cinerea. *Applied and environmental microbiology*, 72(7), 4619–4626.

Siewers, V., Smedsgaard, J., & Tudzynski, P. (2004). The P450 monooxygenase BcABA1 is essential for abscisic acid biosynthesis in Botrytis cinerea. *Applied and environmental microbiology*, 70(7), 3868–3876.

Simon, A. & Biot, E. (2010). ANAIS: analysis of NimbleGen arrays interface. *Bioinformatics (Oxford, England)*, 26(19), 2468–2469.

Singh, A. K., Sharma, V., Pal, A. K., Acharya, V., & Ahuja, P. S. (2013). Genome-Wide Organization and Expression Profiling of the NAC Transcription Factor Family in Potato (Solanum tuberosum L.). *Dna Research*, 20(4), 403–423.

Singh, S., Letham, D. S., & Palni, L. (1992). Cytokinin biochemistry in relation to leaf senescence. VII. Endogenous cytokinin levels and exogenous applications of cytokinins in relation to sequential leaf senescence of tobacco. *Physiologia plantarum*, 86(3), 388–397.

Slusarenko, A. J. & Schlaich, N. L. (2003). Downy mildew of Arabidopsis thaliana caused by Hyaloperonospora parasitica (formerly Peronospora parasitica). *Molecular Plant Pathology*, 4(3), 159–170.

Smale, S. T. & Kadonaga, J. T. (2003). The RNA polymerase II core promoter. *Annual review of biochemistry*, 72, 449–479.

Smedley, D., Haider, S., Ballester, B., Holland, R., London, D., Thorisson, G., & Kasprzyk, A. (2009). BioMart - biological queries made easy. *BMC genomics*, 10, 22.

Smykowski, A., Zimmermann, P., & Zentgraf, U. (2010). G-Box binding factor1 reduces CATALASE2 expression and regulates the onset of leaf senescence in Arabidopsis. *Plant physiology*, 153(3), 1321–1331.

Snyder, C. S., Bruulsema, T. W., Jensen, T. L., & Fixen, P. E. (2009). Review of greenhouse gas emissions from crop production systems and fertilizer management effects. *Agriculture, Ecosystems & Environment*, 133(3), 247–266.

Souer, E., van Houwelingen, A., Kloos, D., Mol, J., & Koes, R. (1996). The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell*, 85(2), 159–170.

Spoel, S. H., Johnson, J. S., & Dong, X. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences of the United States of America*, 104(47), 18842–18847.

Stewart, A. J., Hannenhalli, S., & Plotkin, J. B. (2012). Why transcription factor binding sites are ten nucleotides long. *Genetics*, 192(3), 973–985.

Stewart, A. J. & Plotkin, J. B. (2013). The evolution of complex gene regulation by low-specificity binding sites. *Proceedings. Biological sciences / The Royal Society*, 280(1768), 20131313.

Stotz, H. U., Thomson, J. G., & Wang, Y. (2009). Plant defensins: defense, development and application. *Plant signaling & behavior*, 4(11), 1010–1012.

Stracke, R., Werber, M., & Weisshaar, B. (2001). The R2R3-MYB gene family in Arabidopsis thaliana. *Current Opinion in Plant Biology*, 4(5), 447–456.

Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., Zhou, J.-M., & Chai, J. (2013). Structural basis for flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex. *Science (New York, N.Y.)*, 342(6158), 624–628.

Swartzberg, D., Dai, N., Gan, S., Amasino, R., & Granot, D. (2006). Effects of cytokinin production under two SAG promoters on senescence and development of tomato plants. *Plant biology (Stuttgart, Germany)*, 8(5), 579–586.

Swartzberg, D., Kirshner, B., Rav-David, D., Elad, Y., & Granot, D. (2008). Botrytis cinerea induces senescence and is inhibited by autoregulated expression of the IPT gene. *European journal of plant pathology*, 120(3), 289–297.

Swetlana Friedel, B. U. N. v. W. N. S. (2012). Reverse Engineering: A Key Component of Systems Biology to Unravel Global Abiotic Stress Cross-Talk. *Frontiers in Plant Science*, 3.

Swidzinski, J. A., Sweetlove, L. J., & Leaver, C. J. (2002). A custom microarray analysis of gene expression during programmed cell death in Arabidopsis thaliana. *The Plant Journal*, 30(4), 431–446.

Sýkorová, B., Kurešová, G., Daskalova, S., Trčková, M., Hoyerová, K., Raimanová, I., Motyka, V., Trávníčková, A., Elliott, M. C., & Kamínek, M. (2008). Senescence-induced ectopic expression of the A. tumefaciens ipt gene in wheat delays leaf senescence, increases cytokinin content, nitrate influx, and nitrate reductase activity, but does not affect grain yield. *Journal of Experimental Botany*, 59(2), 377–387.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28(10), 2731–2739.

Taoka, K.-i., Yanagimoto, Y., Daimon, Y., Hibara, K.-i., Aida, M., & Tasaka, M. (2004). The NAC domain mediates functional specificity of CUP-SHAPED COTYLEDON proteins. *Plant Journal*, 40(4), 462–473.

Temme, N. & Tudzynski, P. (2009). Does Botrytis cinerea Ignore H2O2-Induced Oxidative Stress During Infection? Characterization of Botrytis Activator Protein 1. *Molecular Plant-Microbe Interactions*, 22(8), 987–998.

Thomas, H. (2013). Senescence, ageing and death of the whole plant. *New Phytologist*, 197(3), 696–711.

Thomas, H. & Catherine, S. (1993). Crops that stay green1. *Annals of Applied Biology*, 123(1), 193–219.

Thomas, H. & Howarth, C. J. (2000). Five ways to stay green. *Journal of Experimental Botany*, 51 Spec No, 329–337.

Thomas, H. & Ougham, H. (2014). The stay-green trait. *Journal of Experimental Botany*.

Thomas, H. & Stoddart, J. L. (1980). Leaf senescence. *Annual Review of Plant Physiology*, 31(1), 83–111.

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P., & Broekaert, W. F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proceedings of the National Academy of Sciences*, 95(25), 15107–15111.

Thomma, B. P., Eggermont, K., Tierens, K. F.-J., & Broekaert, W. F. (1999). Requirement of Functional Ethylene-Insensitive 2 Gene for Efficient Resistance of Arabidopsis to Infection by Botrytis cinerea. *Plant physiology*, 121(4), 1093–1101.

Thomma, B. P. H. J., Nürnberger, T., & Joosten, M. H. A. J. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant cell*, 23(1), 4–15.

Tiedemann, A. V. (1997). Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with Botrytis cinerea. *Physiological and Molecular Plant Pathology*, 50(3), 151–166.

Tiwari, S. B., Wang, X. J., Hagen, G., & Guilfoyle, T. J. (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *The Plant cell*, 13(12), 2809–2822.

Tomé, D. F. A., Steinbrenner, J., & Beynon, J. L. (2014). A growth quantification assay for Hyaloperonospora arabidopsidis isolates in Arabidopsis thaliana. *Methods in molecular biology (Clifton, N.J.)*, 1127, 145–158.

Tran, L. S. P., Nakashima, K., Sakuma, Y., Osakabe, Y., Qin, F., Simpson, S. D., Maruyama, K., Fujita, Y., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. *The Plant Journal*, 49(1), 46–63.

Tran, L. S. P., Nakashima, K., Sakuma, Y., Simpson, S. D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2004). Isolation and Functional Analysis of Arabidopsis Stress Inducible NAC Transcription Factors That Bind to a Drought Responsive cis Element in the early responsive to dehydration stress 1 Promoter. *THE PLANT CELL ONLINE*, 16(9), 2481–2498.

Tudzynski, P. & Kokkelink, L. (2009). Botrytis cinerea: Molecular aspects of a necrotrophic life style. In *Plant Relationships* (pp. 29–50). Heidelberg: Springer-Verlag.

Ülker, B., Shahid Mukhtar, M., & Somssich, I. E. (2007). The WRKY70 transcription factor of Arabidopsis influences both the plant senescence and defense signaling pathways. *Planta*, 226(1), 125–137.

UN (2014). World Population Prospects - The 2012 Revision.

Usadel, B., Obayashi, T., Mutwil, M., Giorgi, F. M., Bassel, G. W., Tanimoto, M., Chow, A., Steinhauser, D., Persson, S., & Provart, N. J. (2009). Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. *Plant Cell and Environment*, 32(12), 1633–1651.

van Baarlen, P., Woltering, E. J., Staats, M., & van Kan, J. A. (2007). Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three Botrytis species: an important role for cell death control. *Molecular Plant Pathology*, 8(1), 41–54.

van der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flügge, U.-I., & Kunze, R. (2006). Transcription analysis of arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. *Plant physiology*, 141(2), 776–792.

van Kan, J. A. L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science*, 11(5), 247–253.

Van Wees, S. & Glazebrook, J. (2003). Loss of non-host resistance of Arabidopsis NahG to Pseudomonas syringae pv. phaseolicola is due to degradation products of salicylic acid - Van Wees - 2003 - The Plant Journal - Wiley Online Library. *The Plant Journal*, 33(4), 733–742.

Verkest, A., Abeel, T., Heyndrickx, K., Van Leene, J., Lanz, C., Van De Slijke, E., De Winne, N., Eeckhout, D., Persiau, G., Van Breusegem, F., Inzé, D., Vandepoele, K., & De Jaeger, G. (2014). A Generic Tool for Transcription Factor Target Gene Discovery in Arabidopsis Cell Suspension

Cultures Based on Tandem Chromatin Affinity Purification. *Plant physiology.*

Vlot, A. C., Dempsey, D. A., & Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annual review of phytopathology*, 47, 177–206.

Wagner, R., Aigner, H., Pružinská, A., Jänkänpää, H. J., Jansson, S., & Funk, C. (2011). Fitness analyses of Arabidopsis thaliana mutants depleted of FtsH metalloproteases and characterization of three FtsH6 deletion mutants exposed to high light stress, senescence and chilling. *New Phytologist*, 191(2), 449–458.

Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näke, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., & Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant Journal*, 40(3), 428–438.

Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F., Alonso-Blanco, C., Coupland, G., & Albani, M. C. (2009a). PEP1 regulates perennial flowering in Arabis alpina. *Nature*, 459(7245), 423–427.

Wang, X. & Culver, J. N. (2012). DNA binding specificity of ATAF2, a NAC domain transcription factor targeted for degradation by Tobacco mosaic virus. *BMC plant biology*, 12, 157.

Wang, Y., Liu, C., Li, K., Sun, F., Hu, H., Li, X., Zhao, Y., Han, C., Zhang, W., Duan, Y., Liu, M., & Li, X. (2007). Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. *Plant Molecular Biology*, 64(6), 633–644.

Wang, Z., Gerstein, M., & Snyder, M. (2009b). RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews. Genetics*, 10(1), 57–63.

Waters, M. T., Moylan, E. C., & Langdale, J. A. (2008). GLK transcription factors regulate chloroplast development in a cell-autonomous manner. *Plant Journal*, 56(3), 432–444.

Waters, M. T., Wang, P., Korkaric, M., Capper, R. G., Saunders, N. J., & Langdale, J. A. (2009). GLK transcription factors coordinate expression of the photosynthetic apparatus in Arabidopsis. *The Plant cell*, 21(4), 1109–1128.

Weaver, L. M., Gan, S., Quirino, B., & Amasino, R. M. (1998). A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology*, 37(3), 455–469.

Wehner, N., Hartmann, L., Ehlert, A., Böttner, S., Oñate-Sánchez, L., & Dröge-Laser, W. (2011). High throughput protoplast transactivation (PTA) system for the analysis of Arabidopsis transcription factor function. *The Plant Journal*, 68(3), 560–569.

Weir, I., Lu, J., Cook, H., Causier, B., Schwarz-Sommer, Z., & Davies, B. (2004). CUPULIFORMIS establishes lateral organ boundaries in Antirrhinum. *Development (Cambridge, England)*, 131(4), 915–922.

Welner, D. H., Lindemose, S., Grossmann, J. G., Møllegaard, N. E., Olsen, A. N., Helgstrand, C., Skriver, K., & Lo Leggio, L. (2012). DNA binding by the plant-specific NAC transcription factors in crystal and solution: a firm link to WRKY and GCM transcription factors. *Biochemical Journal*, 444(3), 395–404.

Werner, T., Nehnevajova, E., Köllmer, I., Novák, O., Strnad, M., Krämer, U., & Schmülling, T. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and tobacco. *THE PLANT CELL ONLINE*, 22(12), 3905–3920.

Wettenhall, J. M. & Smyth, G. K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics (Oxford, England)*, 20(18), 3705–3706.

Wheeler, T. & von Braun, J. (2013). Climate change impacts on global food security. *Science (New York, N.Y.)*, 341(6145), 508–513.

Williamson, B., Duncan, G. H., Harrison, J. G., Harding, L. A., Elad, Y., & Zimand, G. (1995). Effect of humidity on infection of rose petals by dry-inoculated conidia of Botrytis cinerea. *Mycological Research*, 99(11), 1303–1310.

Williamson, B., Tudzynski, B., Tudzynski, P., & van Kan, J. A. (2007). Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology*, 8(5), 561–580.

Windram, O., Madhou, P., McHattie, S., Hill, C., Hickman, R., Cooke, E., Jenkins, D. J., Penfold, C. A., Baxter, L., Breeze, E., Kiddle, S. J., Rhodes, J., Atwell, S., Kliebenstein, D. J., Kim,

Y.-S., Stegle, O., Borgwardt, K., Zhang, C., Tabrett, A., Legaie, R., Moore, J., Finkenstadt, B., Wild, D. L., Mead, A., Rand, D., Beynon, J., Ott, S., Buchanan-Wollaston, V., & Denby, K. J. (2012). Arabidopsis defense against Botrytis cinerea: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *THE PLANT CELL ONLINE*, 24(9), 3530–3557.

Wingler, A. (1998). Regulation of Leaf Senescence by Cytokinin, Sugars, and Light . Effects on NADH-Dependent Hydroxypyruvate Reductase. *Plant physiology*, 116(1), 329–335.

Wingler, A., Brownhill, E., & Pourtau, N. (2005). Mechanisms of the light-dependent induction of cell death in tobacco plants with delayed senescence. *Journal of Experimental Botany*, 56(421), 2897–2905.

Woo, H. R., Chung, K. M., Park, J. H., Oh, S. A., Ahn, T., Hong, S. H., Jang, S. K., & Nam, H. G. (2001). ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *The Plant cell*, 13(8), 1779–1790.

Woo, H. R., Kim, J. H., Nam, H. G., & Lim, P. O. (2004). The delayed leaf senescence mutants of Arabidopsis, ore1, ore3, and ore9 are tolerant to oxidative stress. *Plant and Cell Physiology*, 45(7), 923–932.

Woody, S. T., Austin-Phillips, S., Amasino, R. M., & Krysan, P. J. (2006). The WiscDsLox T-DNA collection: an Arabidopsis community resource generated by using an improved high-throughput T-DNA sequencing pipeline. *Journal of plant research*, 120(1), 157–165.

Wu, A., Allu, A. D., Garapati, P., Siddiqui, H., Dortay, H., Zanor, M. I., Asensi-Fabado, M. A., Munné-Bosch, S., Antonio, C., Tohge, T., Fernie, A. R., Kaufmann, K., Xue, G.-P., Mueller-Roeber, B., & Balazadeh, S. (2012a). JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. *THE PLANT CELL ONLINE*, 24(2), 482–506.

Wu, X. Y., Kuai, B. K., Jia, J. Z., & Jing, H. C. (2012b). Regulation of Leaf Senescence and Crop Genetic Improvement. *Journal of Integrative Plant Biology*, 54(12), 936–952.

Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., Yang, C., & Xie, Q. (2009). Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Research*, 19(11), 1279–1290.

Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A., & Visser, J. (1999). Cloning and partial characterization of endopolygalacturonase genes from Botrytis cinerea. *Applied and environmental microbiology*, 65(4), 1596–1602.

Xie, Q., Guo, H.-S., Dallman, G., Fang, S., Weissman, A. M., & Chua, N.-H. (2002). SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. *Nature*, 419(6903), 167–170.

Xiong, Y., Contento, A. L., & Bassham, D. C. (2005). AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. *Plant Journal*, 42(4), 535–546.

Xu, B., Ohtani, M., Yamaguchi, M., Toyooka, K., Wakazaki, M., Sato, M., Kubo, M., Nakano, Y., Sano, R., Hiwatashi, Y., Murata, T., Kurata, T., Yoneda, A., Kato, K., Hasebe, M., & Demura, T. (2014). Contribution of NAC Transcription Factors to Plant Adaptation to Land. *Science (New York, N.Y.)*.

Xu, Z.-Y., Kim, S. Y., Hyeon, D. Y., Kim, D. H., Dong, T., Park, Y., Jin, J. B., Joo, S.-H., Kim, S.-K., Hong, J. C., Hwang, D., & Hwang, I. (2013). The Arabidopsis NAC transcription factor ANAC096 cooperates with bZIP-type transcription factors in dehydration and osmotic stress responses. *The Plant cell*, 25(11), 4708–4724.

Yamaguchi, M., Ohtani, M., Mitsuda, N., Kubo, M., Ohme-Takagi, M., Fukuda, H., & Demura, T. (2010). VND-INTERACTING2, a NAC domain transcription factor, negatively regulates xylem vessel formation in Arabidopsis. *THE PLANT CELL ONLINE*, 22(4), 1249–1263.

Yamasaki, K., Kigawa, T., Seki, M., Shinozaki, K., & Yokoyama, S. (2013). DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. *Trends in Plant Science*, 18(5), 267–276.

Yamasaki, K., Kigawa, T., Watanabe, S., Inoue, M., Yamasaki, T., Seki, M., Shinozaki, K., & Yokoyama, S. (2012). Structural basis for sequence-specific DNA recognition by an Arabidopsis WRKY transcription factor. *Journal of Biological Chemistry*, 287(10), 7683–7691.

Yoo, S.-D., Cho, Y.-H., & Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature protocols*, 2(7), 1565–1572.

Yoshida, S., Ito, M., Nishida, I., & Watanabe, A. (2001). Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in Arabidopsis thaliana. *Plant and Cell Physiology*, 42(2), 170–178.

Yoshimoto, K., Jikumaru, Y., Kamiya, Y., Kusano, M., Consonni, C., Panstruga, R., Ohsumi, Y., & Shirasu, K. (2009). Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. *THE PLANT CELL ONLINE*, 21(9), 2914–2927.

Yu, I. C., Parker, J., & Bent, A. F. (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. *Proceedings of the National Academy of Sciences*, 95(13), 7819–7824.

Zarei, A., Körbes, A. P., Younessi, P., Montiel, G., Champion, A., & Memelink, J. (2011). Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the PDF1.2 promoter in Arabidopsis. *Plant Molecular Biology*, 75(4-5), 321–331.

Zdobnov, E. M. & Apweiler, R. (2001). InterProScan–an integration platform for the signature-recognition methods in InterPro. *Bioinformatics (Oxford, England)*, 17(9), 847–848.

Żelisko, A., García-Lorenzo, M., Jackowski, G., Jansson, S., & Funk, C. (2005). AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. *Proceedings of the National Academy of Sciences*, 102(38), 13699–13704.

Zhang, K. & Gan, S.-S. (2012). An abscisic acid-AtNAP transcription factor-SAG113 protein phosphatase 2C regulatory chain for controlling dehydration in senescing Arabidopsis leaves. *Plant physiology*, 158(2), 961–969.

Zhang, K., Xia, X., Zhang, Y., & Gan, S.-S. (2012a). An ABA-regulated and Golgi-localized protein phosphatase controls water loss during leaf senescence in Arabidopsis. *The Plant Journal*, 69(4), 667–678.

Zhang, L., Li, Z., Quan, R., Li, G., Wang, R., & Huang, R. (2011). An AP2 domain-containing gene, ESE1, targeted by the ethylene signaling component EIN3 is important for the salt response in Arabidopsis. *Plant physiology*, 157(2), 854–865.

Zhang, P. & Gruissem, W. (2005). Leaf senescence-Inducible expression of isopentenyl transferase in cassava rendering it resistant to drought stress. (pp. 24–28).

Zhang, P., Wang, W. Q., Zhang, G. L., Kamínek, M., Dobrev, P., Xu, J., & Gruissem, W. (2010). Senescence-Inducible Expression of Isopentenyl Transferase Extends Leaf Life, Increases Drought Stress Resistance and Alters Cytokinin Metabolism in Cassava. *Journal of Integrative Plant Biology*, 52(7), 653–669.

Zhang, W., Zhang, T., Wu, Y., & Jiang, J. (2012b). Genome-Wide Identification of Regulatory DNA Elements and Protein-Binding Footprints Using Signatures of Open Chromatin in Arabidopsis. *The Plant cell*, 24, 2719–2731.

Zhang, Z., Qin, G., Li, B., & Tian, S. (2014). Knocking Out Bcsas1 in Botrytis cinerea Impacts Growth, Development, and Secretion of Extracellular Proteins, Which Decreases Virulence. *Molecular Plant-Microbe Interactions*, 27(6), 590–600.

Zhong, R., Demura, T., & Ye, Z.-H. (2006). SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *The Plant cell*, 18(11), 3158–3170.

Zhong, R., Richardson, E. A., & Ye, Z.-H. (2007a). The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. *The Plant cell*, 19(9), 2776–2792.

Zhong, R., Richardson, E. A., & Ye, Z.-H. (2007b). Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. *Planta*, 225(6), 1603–1611.

Zhou, X., Jiang, Y., & Yu, D. (2011). WRKY22 transcription factor mediates dark-induced leaf senescence in Arabidopsis. *Molecules and cells*, 31(4), 303–313.

Zhu, Q., Zhang, J., Gao, X., Tong, J., Xiao, L., Li, W., & Zhang, H. (2010). The Arabidopsis AP2/ERF transcription factor RAP2. 6 participates in ABA, salt and osmotic stress responses. *Multiple values selected*, 457(1-2), 1–12.

Zimmermann, P. & Zentgraf, U. (2005). The correlation between oxidative stress and leaf senescence during plant development. *Cellular & molecular biology letters*, 10(3), 515–534.

Zwack, P. J. & Rashotte, A. M. (2013). Cytokinin inhibition of leaf senescence. *Plant signaling & behavior*, 8, 24737e.